MOLECULAR ANALYSIS OF WET ROT ORGANISMS; APPLICATION TO CONSERVATION OF MARITIME ARTIFACTS.

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

Signed [redacted] Date 14/12/92..

(Director of Studies)

THIS MANUSCRIPT IS DEDICATED TO MY BEST FRIEND, FRASER.

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MOLECULAR ANALYSIS OF WET ROT ORGANISMS; APPLICATION TO CONSERVATION OF MARITIME ARTIFACTS.

HELEN M^{C}DOWELL

ABSTRACT.

Conventional identification of decay fungi is time consuming and requires a knowledge of taxonomy. Identification systems were therefore developed using SDS-PAGE and western blotting. The production of unique protein and antigen profiles for the *Coniophora* genus using whole cell and exoprotein extracts, allowed the differentiation of *Coniophora* organisms from other fungi known to inhabit wood. In addition intra- and inter-species variation of *Coniophora* antigens was evident, indicating that identification of individual strains and species of *Coniophora* is possible. Further, antigenic variation within the strains of *C. puteana* suggested that organisms within the species *C. puteana* may have been misclassified.

The potential of the systems developed to identify other decay fungi was tested by the study of organisms from maritime artifacts present in Dundee. The systems allowed positive identification of some isolates, whilst other isolates now require further analysis. A large range of decay fungi was found on these ships, whilst the predominant organism colonising the timbers of the *Unicorn*, was *Coniophora marmorata*. Consequently detection systems initially developed for *C. puteana* were also required to detect *C. marmorata*.

Currently relatively unsophisticated but effective methods of detecting decay in maritime artifacts are utilised but only detect decay once substantial structural damage has occurred. The potential of immunological methods for the *in situ* detection of antigens of *C. puteana*, prior to the structural damage of the timber was therefore investigated. Polyclonal antisera produced against this organism, allowed the detection of the organism in laboratory decayed wood, but the antisera were highly cross-reactive towards other decay fungi. These antisera although of limited use for detection, provided useful information on the antigenicity and immunogenicity of various mycelial extracts of *C. puteana*. Consequently, monoclonal antibodies were produced against an exoantigen extract of *C. puteana*.

The monoclonal antibody probe developed reacted with other members of the *Coniophora* genus, allowing detection of *C. marmorata* in field samples from the *Unicorn*. However further cross-reactivity studies indicated that this probe was not specific to the *Coniophora* genus. Consequently the use of this probe for the specific detection of *C. marmorata* in the field, is only possible when used in conjunction with SDS-PAGE and western blotting.

CONTENTS PAGE

ABBREVIATIONS

ABBREVIATION		DEFINITION
cm	-	centimetre
cm^3	-	centimetre cubed
°C	-	degrees centigrade
Da	-	daltons
g	-	gram
h	-	hour
kDa	-	kilodaltons
M	-	molar
mA	-	milliamps
mg	-	milligram
min	-	minute(s)
ml	-	millilitre
mM	-	millimolar
mm	-	millimetre
nm	-	nanometres
ppm	-	parts per million
rpm	-	revolutions per minute
v/v	-	volume by volume
w/v	-	weight by volume
µg	-	microgram
µl	-	microlitre
µm	-	micrometres
A	-	agar
Ab	-	antibody
Acryl	-	acrylamide
Ag	-	antigen
Bisacryl	-	bisacrylamide
BSA	-	bovine serum albumin
C	-	cellulose
CAT	-	modified computed axialtomography
CCA	-	copper-chrome arsenic
cf	-	compared to
CR	-	cross-reactivity
DAB-NiCl	-	diaminobenzidine-nickel chloride
DIT	-	Dundee Institute of Technology
EA	-	exoantigen
EAV	-	Vigrow, 1992
EDTA	-	ethylenediamine tetra-acetic acid
EIA	-	enzyme immunoassay
EP	-	exoprotein
FCS	-	foetal calf serum
FD	-	freeze dried
GT	-	growing tip
HAT	-	hypoxanthine-aminopterin-thymidine
HRP	-	horseradish peroxidase
HT	-	hypoxanthine-thymidine
Hwb	-	high weight loss block
ID	-	immunodiffusion
IE	-	immunoelectrophoresis
IF	-	immunofluorescence
IgG	-	immunoglobulin gama
IMS	-	ion mobility spectrometry

ip	-	intraperitoneal
IR	-	infrared
Lwb	-	low weight loss block
MAb(s)	-	monoclonal antibody/antibodies
MWM	-	standard molecular weight markers
MX	-	malt extract
MXB	-	malt extract broth
NCS	-	newborn calf serum
NMS	-	normal mouse serum
No	-	number
NRS	-	normal rabbit serum
NRtS	-	normal rat serum
o/n	-	over night
PBS	-	phosphate buffered saline
PEG	-	polyethylene glycol
REF	-	reference
res gel	-	resolving gel
RIA	-	radio-immunoassay
RRS	-	Royal Research Ship
RT	-	room temperature
S	-	sawdust
SAPU	-	Scottish Antibody Production Unit
sc	-	subcutaneous
SDS-PAGE	-	sodium dodecylsulphate polyacrylamide gel electrophoresis
s/n	-	supernatant
stack gel	-	stacking gel
SW	-	surface washings
TBS-T	-	tris buffered saline
TBTO	-	tri-butyl tin oxide
TEMED	-	N,N,N,N-tetramethylethylenediamine
TMB	-	tetramethyl benzidine
TnBTN	-	tri-n-butyl tin naphthenate
tw	-	tween 20
U	-	*Unicorn*
UCi	-	*Unicorn* core isolate
wk	-	week
WM	-	whole mycelia
wt	-	weight

ORGANISMS

AX	-	*Amyloporia xantha*
B15	-	*Coniophora puteana*
CA	-	*Coniophora arida*
CM	-	*Coniophora marmorata*
Cp	-	*Ceratocystis picea*
CP	-	*Coniophora puteana*
CR	-	*Cladosporium resinae*
CV	-	*Coriolus versicolor*
DC	-	*Daldinia concentrica*
DQ	-	*Daedalea quercina*
FV	-	*Fibroporia vaillantii*
GS	-	*Gloeophyllum sepiarium*
GT	-	*Gloeophyllum trabeum*
HA	-	*Heterobasidion annosum*

HP	-	*Hyphoderma puberum*
LL	-	*Neolentinus (Lentinus) lepideus*
LS	-	*Laetiporus sulphureus*
PG	-	*Peniophora gigantea*
PI	-	*Poria incrassata*
PIg	-	*Phellinus igniarius*
Pp	-	*Paxillus panuoides*
PO	-	*Pleurotus ostreatus*
PP	-	*Poria placenta*
PV	-	*Paeciliomyces variotii*
SC	-	*Schizophyllum commune*
SL	-	*Serpula lacrymans*
SP	-	*Leucogyrophana (Serpula) pinastri*
SS	-	*Stereum sanguinolentum*
TH	-	*Trichoderma harzianium*
TP	-	*Trichoderma polysporium*
V	-	*Verticillium*
VLe	-	*Verticillium lecanii*
VL	-	*Verticillium lamellicola*
11A	-	*Coniophora puteana* FPRL 11A
11B	-	*Coniophora puteana* FPRL 11B
11E	-	*Coniophora puteana* FPRL 11E
11Q	-	*Coniophora puteana* FPRL 11Q

CHAPTER 1

INTRODUCTION

1.0. INTRODUCTION

All substrates containing cellulose can be naturally decomposed by the action of cellulolytic enzymes produced by microorganisms. Wood in the living tree, or in all types of service, including wooden ships, can therefore be degraded by decay fungi, if the conditions are conducive for colonisation. Often, the ensuing damage causes undesirable ecomomic problems. Current methods to detect such decay only provides evidence of colonisation of the wood, once substantial decomposition has occurred. Novel methods to overcome the lack of recognition of the onset of decay are desired and require to possess the ability to detect the presence of wood decay fungi prior to substantial destruction of the wood, subsequently allowing the appropriate preservation treatment to be applied to eliminate the undesired microbes. The most promising techniques to date, for such detection are molecular identification methodologies and immunodetection assays.

The studies described in this thesis examine molecular and immunological methods for the identification and detection of the major wet rot organism *Coniophora puteana* in wood and the application of the systems developed to the timbers of maritime artifacts.

1.1. THE LIVING TREE

In the standing tree, wood like any living material has its own mechanisms for defence. The physical barrier is the bark of the tree which protects the inner tissue against wounding by insects, animals or birds and against entry of

infectious fungal or bacterial spores. When the bark is damaged, an immediate reaction in many ways similar to an immune response in animals is initiated. This "immune response" is of two kinds; compartmentalisation of the damaged tissue and the production and release of chemical from the wood cells. Compartmentalisation involves the development of a "protective box" around the wounded tissue. The enclosed area has four basic wall types and is shown in Figure 1.1. Wall 1 is formed by the plugging of xylem vessels by cambium and parenchyma cells above and below the location of the wound; wall 2 is formed by existing annual rings; wall 3 is formed by medullary rays and wall 4 by a barrier zone which is produced between wood present at the time of wounding and wood subsequently formed (Mercer, 1982). The chemicals which are produced and released are specific fungistatic or insecticidal wood extractives such as phenols and terpenes which may or may not be released in association with compartmentalisation (Gramss, 1987). Compartmentalisation and chemical release are not limited to stems and branches. Both have been observed in roots of red pine (*Pinus resinosa*) after invasion by the tree pathogen *Heterobasidion annosum* (Shigo, 1975). Copious amounts of resin have also been noted exuding from the resin canals of wounded conifers as a result of both compartmentalisation and chemical release or as a result of these responses occurring individually (Mercer, 1982). In broadleaved trees the production of polyphenols as a chemical barrier has been observed and Malterud *et al.* (1985) reported that the wood of the willow tree *Salix caprea* produces flavenoids which inhibit the growth of wood destroying fungi.

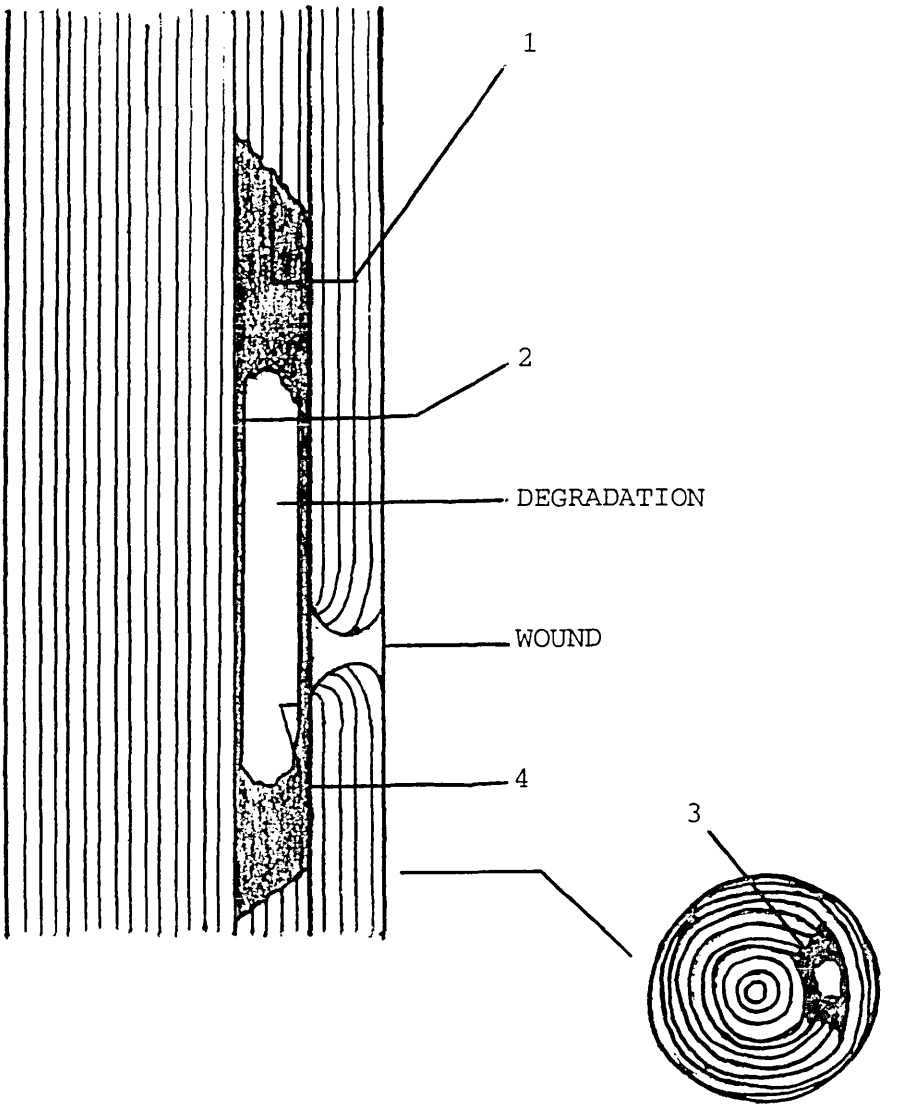

FIGURE 1.1. DIAGRAMMATIC ILLUSTRATION OF COMPARMENTALISATION PRODUCED BY FOUR WALL TYPES

1.2. TIMBER: ITS SUSCEPTIBILITY TO HOST INVASION

In living trees the sapwood is much more resistant to decay than the heartwood. This however changes dramatically with death or felling of the tree and its subsequent use (Dickinson, 1982). The "immune response" of the sapwood to host infection is completely lost, and the wood becomes a target for degradation by mechanical wear, chemical stress and biodegradation by insect and microbial attack (Cartwright and Findlay, 1958). Biodegradation is by far the most important of these three forms of stresses particularly for timber in storage, in domestic buildings and in wooden ships.

Insect invasion of timber is the cause of a great deal of damage to structures in tropical countries. In temperate climates however damage to timber by insects in storage and in service causes less economic loss than fungal decay (Findlay, 1975).

1.2.1. TIMBER: INFECTION IN STORAGE

After harvesting, logs and large timbers may take many months to air-dry (season) and during this time much internal decay can occur, either continuing from infection in the standing tree or from new infection. The sapwood being no longer resistant to decay can rapidly become subject to natural microbial colonisation by bacteria, mould and staining fungi and lastly wood-destroying fungi. Although the natural defence mechanisms of the wood are lost, protection in the short term is afforded by the intrinsic microbial succession described, the decay fungi

representing the last organisms in the degradative sequence (Gramss, 1987).

In order to prevent decay of logs and large dimension timber, harvest in the winter is required when the temperature is lower than the optimum for fungal growth and storage anaerobically under water is necessary. Prevention of decay of sawn timber is by seasoning. This involves the air-drying of timber to prevent the colonisation of microorganisms in winter or by the direct use of kiln drying immediately after felling and sawing. Imported timber is either air-dried immediately or is dipped in antiseptic solutions prior to shipping (Cartwright and Findlay, 1958).

1.2.2. TIMBER: INFECTION IN BUILDINGS

The growth of wood decay organisms in building timbers depends wholly on the physical condition of the building timbers. The necessary state of timber for fungal proliferation is detailed below;

a. High moisture content of the wood,

b. Oxygen availability,

c. Nitrogen availability, and

d. Absence of toxic chemicals.

A low moisture content is of critical importance for the durability of timber, particularly in buildings. Dickinson

(1982) quoted a figure of £3,000,000 per week for repairing damage caused by wood-rotting fungi in buildings in 1977; all of this damage was attributed to water ingress. The prevention and cure of decay within buildings is subsequently linked to the understanding of building problems such as water ingress, rising damp, ineffective sub-floor ventilation, condensation, general rainware and plumbing problems. The attack of external building joinery by wood-decay fungi is similarly due to the penetration of water into open joints such as window joinery as a result of design and manufacturing faults.

1.2.3. TIMBER: INFECTION IN WOODEN SHIPS

The problems of fungal decay of in-service timber is particularly amplified in buildings, as previously described. In historic times however the decay of ships was of major economic and political importance and because of this the history of decay in ships is well documented. Prior to 1609 there were few references of degradation which indicated whether the life of the ships were reduced by insect attack or by fungal decay. However, the insect *Teredo navalis* was found to be responsible for the destruction of Sir Francis Drake's ship *Golden Hind* in the sixteenth century and the HMS *Victory* of the eighteenth century was subject to severe attack by insects which multiplied in the thick timbers of the hull (Allsopp and Seal, 1986; Findlay, 1975).

Fungal deterioration of wooden ships was a major economic problem and documented evidence is available to the present day. In the seventeenth century the problem of decay was

noted and a "Naval Commission of Inquiry" was appointed by James I in 1609. In the report sound advice to replace green timber by seasoned timber was given but was subsequently ignored and "fleet after fleet rotted prematurely" (Ramsbottom, 1937). This kind of ignorance was repeated constantly, for example, during the Napoleonic wars, the increased demand for ships led to the use of unseasoned wood which coupled with lack of ventilation had catastrophic effects. As a result many of these ships became decayed by fungi and needed extensive repair soon after launching. Ramsbottom (1937) quotes

> "the duration of a ship was estimated at 25-30 years in the seventeenth century, about 12 years from 1760-1788, about 8 years during the Napoleonic period, dwindling to no duration immediately after Trafalgar (1805)".

The economic loss from such destruction and the cost of preventative treatment were enormous. Specific examples are, the 64-Gun *Ardent* which was launched in 1764 at a cost of £23,000. She had a repair bill of more than half her original cost within seven years (Goodwin, 1987); most notable to historians because of the graphic descriptions available was the *Queen Charlotte*, launched in 1810 at a cost of £88,534. She was severely infected with the dry rot fungus *S. lacrymans* and needed to be repaired even before going to sea; within six years the repair bill had amounted to more than her original cost (Ramsbottom, 1937).

As a result of such widespread economic losses, a range of preservative treatments were attempted to prevent hull deterioration. The actual construction of ships were also modified to allow sufficient ventilation of timbers. The

most successful method of preserving the hulls of ships was found to be the incorporation of an outer copper sheathing over the timbers of the hull. This became general practice in 1782 (Goodwin, 1987). Other methods included the pickling of timbers in brine prior to their incorporation into the ship. This was introduced by Robert Seppings at the beginning of the nineteenth century (Goodwin, 1987).

1.2.4. HISTORY OF MARITIME ARTIFACTS

Surprisingly some wooden ships of the vintages described are still intact and there is now increasing attention given to the conservation of maritime artifacts. Below is a brief account of the type of ships which have survived from various eras and are presently on show in maritime museums.

a. The *Mary Rose* (1510) of the Tudor Period is now exhibiting her ancient oak timbers at Portsmouth, after being rescued from the sea in 1982 where she lay since 1545.

b. The *Victory* (1765) constructed in the eighteenth century is a prime exhibit of the skills of Georgian shipwrights and is presently on show at the Naval museum in Portsmouth.

c. The *Foudroyant* constructed in 1817 is also an example of a Georgian-built ship and is now known as the *Trincomalee* which was her original name. Presently, she is under the protection of the *Trincomalee* Trust, in Hartlepool.

d. The frigate *Unicorn* (1824) presently on show at Dundee under the protection of The *Unicorn* Preservation Society, was constructed at the transition between wooden-clad and iron-clad ships at the beginning of the nineteenth century.

e. The *Gannet* (1878) provides history of the steam engine and screw propeller and is presently undergoing restoration at Chatham dockyard, Kent.

f. The HMS *Warrior* (1861) was the Royal Navy's first iron-clad warship and is currently displayed at the Naval museum in Portsmouth.

g. The Royal Research Ship (RRS) Discovery (1901) is an example of an exploration ship and exhibits the type of construction required to withstand pressure from the ice of Antarctica. Currently the *Discovery* is undergoing restoration in Dundee.

Two historic ships briefly described above have become part of the current study viz., the frigate *Unicorn* and the RRS *Discovery*, since they are both subject to decay by wet rot fungi. Detailed descriptions of these ships are given in Chapter 7.

1.3. ORGANISMS ASSOCIATED WITH WOOD DECAY

A wide variety of organisms can decay in service timber whether in ships or in buildings and these organisms are detailed below.

The initial colonisers of felled wood are bacteria. The attack of wood cells proceeds very slowly as bacteria cannot grow in size, their expansion being determined by cell division. Invasion occurs through the ray parenchyma cells although random distribution is also observed, thus increasing the porosity of the wood cells allowing further colonisation of microbes (Fengel and Wegner, 1984). Subsequent colonisers are the staining and mould fungi, which belong to the Ascomycetes and Fungi Imperfecti (Deuteromycetes) groups. These fungi do not cause substantial decay of wood, but they do reduce the aesthetic value of the timber by staining caused by the deposition of dark material in vacuoles within the hyphae (Levy, 1982; Fengel and Wegner, 1984). Due to the staining produced, these fungi may act as useful indicators of damp conditions. The hyphae of the fungi belonging to the staining and mould groups, grow mainly in the parenchyma cells living on the proteinaceous content, but are also found in tracheids where they grow on the inner cell wall surface without an enzymic alteration of the cell wall structure (Fengel and Wegner, 1984). The ultimate colonisers are the most important microbial group in relation to wood decay. These wood destroying organisms belong to the subdivisions Ascomycete and Deuteromycete (soft rot fungi), and Basidiomycete (white and brown rot fungi) (Ross, 1983). The location of these fungi in the wood cell wall is detailed in Figure 1.2.

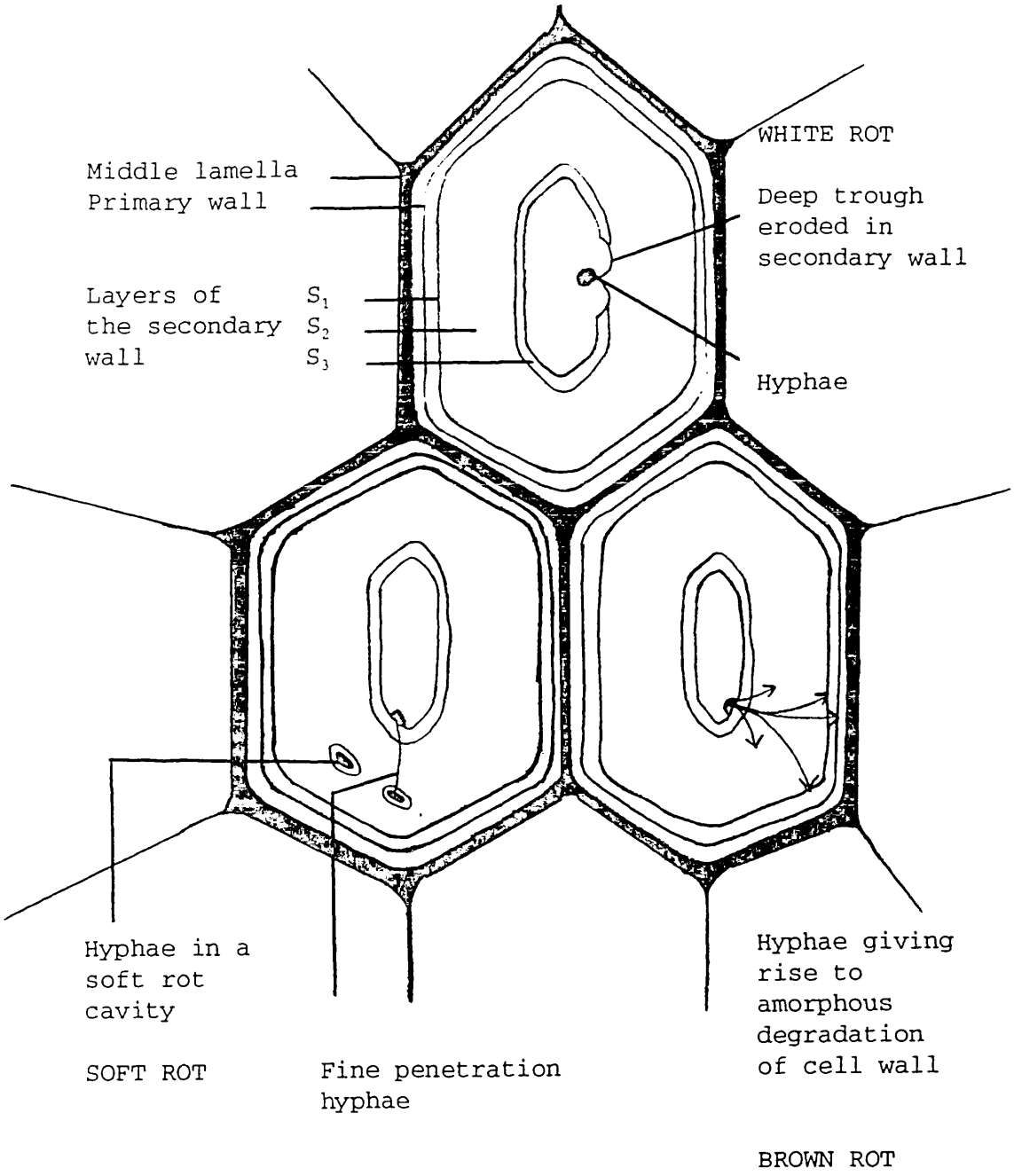

FIGURE 1.2. DIAGRAMMATIC REPRESENTATION OF FUNGAL INVASION OF THE WOOD CELL WALL (Montgomery, 1982).

The Basidiomycetes have a greater potential to decay wood than other fungal organisms since they,

a. possess effective dispersal mechanisms,

b. require a relatively low moisture content of the wood,

c. possess the ability to penetrate the cell walls of wood,

d. possess a highly effective enzyme complement system for the degradation of the components of wood,

e. grow in size, therefore proliferation is speedy, and they,

f. transport nutrients via their advanced hyphal network.

The members of this family are divided according to the enzymes which they secrete. White rot fungi secrete cellulases and ligninases resulting in loss of both the cellulose and lignin components of the cell wall. Consequently, the wood becomes bleached and splinters (Technical Note No. 44, FPRL). Brown rot fungi, however secrete only cellulases (for details, see 1.5.), thus losing the cellulose but not the lignin component of the cell (Coggins, 1980). The brown rot fungi frequently occur in buildings throughout Europe and are divided into two types of rot. The first type is that caused by the true dry rot fungus *Serpula lacrymans* and the second is that caused

by wet rot fungi, the most common of which is *Coniophora puteana*. Both of these fungi belong to the family *Coniophoraceae*.

1.4. THE *CONIOPHORACEAE* FAMILY

The *Coniophoraceae* is a family of the order *Aphyllophorales* (Ginns, 1982 and Hudson, 1986). It is a small family both in the number of genera (6) and total number of species (30-35). Nearly all of the species are lignicolous and are known to cause brown rot of wood and 15 species of the family can degrade pure cellulose. Within the family *Coniophoraceae* are included the genera *Coniophora*, *Serpula*, *Leucogyrophana* and *Coniophorella* (Rayner and Boddy, 1988). The general circumscription of the genus *Coniophora* has remained constant for over a century and is composed of 5 common species and 10 species that have been infrequently or rarely collected. The species of the genus *Coniophora* were primarily isolated on coniferous wood mostly in cool temperature regions. All species cause a brown rot of wood and some are often or exclusively known from domestic habitats (Ginns, 1982).

The complete geographic distribution of the species of *Coniophora* is not known because of the lack of mycological surveys in many regions and countries. However a few species are known from a sufficient number of localities and collections to allow a pattern of distribution to discerned. A review of this distribution was produced by Ginns (1982). It covered six regions of the world and indicated that the most cosmopolitan species of the genus *Coniophora* is *C. puteana* which is known from the following

continents: North America, South America, Europe, Africa, Asia and Australasia. In Europe 5 *Coniophora* species are considered to be widespread, *C. puteana*, *C. arida*, *C. fusispora*, *C. olivacea* and *C. marmorata*. The occurrence of the species *C. marmorata* has only recently been discussed in European literature, however it is probable that *C. marmorata* has a wider distribution than is presently known. Since it occurred in domestic habitats, it may have been confused with the better known *C. puteana* (Ginns, 1982).

The members of the genus *Coniophora* have their most direct effect on domestic life due to their ability to destroy structural timbers in buildings (Cartwright and Findlay, 1958). The economic impact is seen in the structural weakening of timber which if severe requires removal and replacement. In Britain the most important species of the genus *Coniophora* causing decay of in service timber is *C. puteana*. *C. puteana* was reported to be responsible for decay in solid floors laid on to concrete without adequate damp-proof course (Findlay, 1937). It is however most commonly found infecting the structural timbers of buildings which have become soaked by leakage of water, for example, floorboards, window joinery and roofing timbers (Coggins, 1980). *C. puteana* has also been described as a primary parasite in living trees, for example, *Sequoia gigantea* (redwood) (MacDonald, 1939), and spruce and pine trees in Norway (Jorstad and Juul, 1939). Although *C. puteana* can still be isolated from the standing tree today, it is not a major tree pathogen (Gramss, 1985). *C. puteana* was perhaps a source of infection of timber subsequently used in construction in the late 1930's.

1.5. *C. PUTEANA*

C. puteana is a basidiomycete organism which preferentially attacks softwoods, but may also attack woods such as western red cedar and the durable jarrah (Cartwright and Findlay, 1958). The primary mycelia of *C. puteana* have uninucleate compartments bound by simple septa and are defined as homokaryotic. They also possesses clamp connections. Ainsworth and Rayner (1986 and 1989) and Beeching *et al.*, (1989), have shown that despite having clamp connections, homokaryotic primary mycelia can outcross. That is, they will interact in mating type-compatable combinations to produce distinct secondary mycelia with binucleate compartments (mating-type secondary heterokaryons). Ainsworth and Rayner, (1990) reported that this is also the case for the homokaryotic primary mycelia of *C. puteana*. The production of the stable secondary heterokaryon between outcrossing homokaryons (primary mycelia) is possible. However there exists a fine control between rejection (heterogenic incompatability) and acceptance (homogenic incompatability or more logically homogenic compatability; Rayner *et al.*, 1984) of hyphal contents. The rejection response is stimulated by the presence of genetically non-alike individuals. This response can be overridden by the presence of complementary mating factors resulting in outcrossing between genetically different cell lines. The action of the mating factors allows the exchange of genetic material to occur resulting in the production of the secondary heterokaryon. Complete or partial lack of functioning of the override system allows expression of the rejection response resulting in a

termination of fusion of secondary heterokaryons, thus maintaining the species (Ainsworth and Rayner, 1990).

The distribution of *C. puteana* starts by the liberation of small yellow/brown spores from the reproductive heterokaryotic basidiocarp (Ginns and Kokko, 1976). The germination of these ellipsoid spores is initiated when the wooden substrate is at or above fibre saturation point, a moisture content of 26-32% (Hudson, 1986; Viitanen and Ritschkoff, 1991). It is generally recognised that relatively high moisture levels are required for infection of wood by *C. puteana*. However there is some disagreement about absolute levels. Lehmann and Scheible (1923) reported that *C. puteana* requires a high moisture content of 50-60% for growth. However, the work of Ferdinandsen and Buchwald (1937) suggested that the maximum decay of wood by this organism occurred at a moisture content of 34-36%, which is just above the fibre saturation point of the wood, at which point germination can occur. Viitanen and Ritschkoff (1991), have since reported that the optimum wood moisture content for *C. puteana* is 30-70%, since the moisture content of the wood varies with the growth and activity of the organism.

Once spore germination has occurred homokaryotic primary mycelia emerge from the spore and outcross to produce heterokaryotic secondary mycelia. The fungus moves into the wood cell lumena where there is access to cellulose (MacDonald, 1939 and Ginns, 1982), mechanically via bordered pits and enzymically by erosion to produce bore holes (3.5µm diameter; Cartwright and Findlay, 1958) which are somewhat wider than the hyphae (3-10µm diameter;

Rattan, 1977). *C. puteana* causes the wood to darken producing a brown rot in which wall polysaccharides are principally utilised. Though little of the lignin is used, it may be altered structurally by the removal of methoxyl groups. There is no thinning of the cell wall and the enzymes and other destructive chemicals responsible diffuse away from the hyphae and act on the entire cell wall at some distance from the hyphae. The structural polymers are removed leaving a framework of lignin to maintain the general shape so that there is little apparent damage until the cell walls collapse. Decomposition occurs in irregular patches in the attacked area, leading to the cuboidally cracked appearance characteristic of brown-rotted wood, cracks along the grain often being concealed by a thin layer of sound wood (Cartwright and Findlay, 1958).

C. puteana has the ability to "self-wet" wood during the decay process. The degradation of cellulose produces carbon-dioxide and water and this metabolic water which is independent of any external supply, amounts to approximately 0.56g for every 1g cellulose decomposed (Hudson, 1986 and Miller, 1933). Therefore *C. puteana* has the inherent ability to survive once established in wood, although the moisture content may fall slightly below the fibre saturation point of the wood. *C. puteana*, is however sensitive to desiccation, the activity of the organism ceasing as soon as moisture is removed. The optimum temperature for growth of *C. puteana* is 23°C and the activity of the organism ceases at temperatures at or above 35°C (Cartwright and Findlay, 1958). The high temperatures common in Africa, may be an explanation for the lower occurrence of *C. puteana* in this region. The growth rate of

C. puteana is reported as 13.5mm per day on agar medium and the decay of Scots pine sapwood (blocks of dimensions 5x2.5x1.5cm)
at 22°C over four months resulted in a loss of 40% of the original dry weight of the blocks (Theden and Schulze, 1942).

1.5.1. DECAY BY *C. PUTEANA*

The chemical and biological mechanisms of the decay process of *C. puteana* are still unclear although the overall method of attack is by the depolymerisation (DP) of cellulose and the modification of lignin. There seem to be three possible modes of attack of wood by *C. puteana*, and most probably other basidiomycetes. These three areas are enzymic degradation (Schmidhalter and Canevascini, 1990), the peroxide/iron system of decay suggested by numerous researchers (Enoki *et al.*, 1991 and, Micales and Highley, 1987) and chemical attack, for example acids released from the hyphae during growth (Birkinshaw *et al.*, 1940).

1.5.1.1. ENZYMIC ATTACK OF WOOD

The production of extracellular cellulolytic enzymes by wood decay fungi is associated with wood decomposition (Highley, 1988). The complete cellulolytic enzyme complement which is responsible for this degradation is present in the white rot fungi but only some brown rot fungi contain the complete complement, for example members of the *Coniophoraceae* family, viz., *C. puteana* and *S. lacrymans* (Highley, 1988). The cellulase complex consists of endo-β-1,4-glucanase (Cx complex), exoglucanase (C1

complex or cellobiohydrolase) and β-glucosidase (Ritschkoff and Viitanen, 1989) along with endocellulase, mannase and xylanase. The suggested mode of attack by this cellulolytic system is the hydrolysis of the 1,4-β-linkage of crystalline cellulose by the endo-β-1,4-glucanase and exoglucanase to produce cellobiose. This is then cleaved by β-glucosidase to liberate glucose which is subsequently decomposed to carbon-dioxide and water by Krebs' cycle enzymes (Ritschkoff and Viitanen, 1989). Schmidhalter and Canevascini (1990), reported the presence of two cellobiohydrolases and a cellobiose-dehydrogenase, which are released from the hyphae of *C. puteana* during the degradation of cellulose. This further confirms that *C. puteana* does possess the full cellulolytic system and it can be shown to degrade crystalline cellulose unlike some other fungi. However, the method of entrance of these cellulolytic enzymes into the wood cell wall is still uncertain. One suggestion by Flournoy *et al.*, (1991), was the opening of the cell wall structure of the wood after the intitial attack of wood decay organisms, giving access to enzymes. However he subsequently disproved this theory. The objectives of his study were to determine the following;

a. the pore volume in the sound wood cell wall available to molecules of different size,

b. whether attack by a brown rot fungus during which cellulose is depolymerised, results in a sudden increase in cell wall volume, and

c. whether this decay opens up the pore structure of the cell wall in a manner that allows access by large molecules.

His first finding was that the actual pore size of seasoned wood (15Å) was much smaller than previously reported (100Å) because previous workers had used "never-dried" or green wood. On decay the accessible wall volume increased only slightly if at all for the accessibility of large molecules (>38Å). For the entrance of small molecules (<12Å), the volume increased to a small extent suggesting a slight increase in the size of existing pores. Flournoy concluded that the decay due to brown rot fungi does not open up the pore structure to give access to large molecules; decay does not suddenly increase the cell wall volume and the pore volume is only accessible to small molecules in both sound and decayed wood. Other changes may be responsible for enzyme access, for example mechanical disruption of the wood cell wall, possibly by the hyphae themselves.

The hyphae of many basidiomycetes including *C. puteana* are known to possess an extracellular layer which envelopes the vegetative hyphae (a hyphal sheath) (Palmer, 1983). This sheath, which has been studied for a number of fungi including the brown rot fungus *Poria placenta* (Green *et al.*, 1989), is thought to be the site and pool of fungal enzymes. It contains extracellular membranous structures which assume a variety of forms including lamellar sheets, fibrils and vesicles. These structures were found on the surface of hyphae, extending from hyphae on to wood surfaces and covering the S3 layer, embedded in the hyphal sheath and penetrating wood cell wall layers. The purpose

of the hyphal sheath has been reported by Palmer (1983) and Green *et al.*, (1989), to,

a. provide attachment to solid substrates,

b. act as a nutrient reserve,

c. provide protection against toxic chemicals,

d. facilitate wood degradation by storing or concentrating wood degrading agents,

e. maintain optimum pH for enzyme activity and,

f. condition the substrate prior to enzyme action.

1.5.1.2. DECAY BY PEROXIDE/IRON SYSTEM

Although *C. puteana* has been shown to produce the full enzyme complement for the degradation of cellulose, further or alternative deterioration by the peroxide/iron system of decay has been postulated (as discussed below), as a likely cause of the primary breakdown of the cellulose matrix by brown rot fungi. Furthermore those basidiomycetes which do not possess the full enzyme complement for the degradation of cellulose, for example *P. placenta* (Highley, 1980), must possess an alternative method for the initial decomposition of cellulose; again the peroxide/iron system has been implicated.

The peroxide/iron system involves the production and utilisation of hydrogen peroxide, by fungal organisms,

which in combination with iron II (present in wood) aids in the DP of cellulose (Koenigs, 1974). Low concentrations of H_2O_2 and Fe II allow DP of cellulose in wood to occur, but neither H_2O_2 or Fe II alone can cause substantial degradative effects on cellulose in wood, even at high concentrations. The actual mechanisms of decay of wood by the peroxide/iron system are uncertain, but it is known (Montgomery, 1982) that it is an oxidative process involving the production of free oxy- and hydroxy-radicals. Enoki *et al.*, (1990), have suggested that brown rot fungal hyphae in the cell lumen can extracellularly secrete compounds which can diffuse freely from the hyphae to penetrate deeply into the microstructure of the wood cell wall. These compounds which are glycoproteins of molecular weights of ~1,600-2,000 Daltons, chelate Fe II from the wood and with H_2O_2 cause the production of oxy- and hydrox- radicals which consequently demolymerise cellulose. This proposed mechanism for the DP of cellulose is detailed below with reference to Figure 1.3.

The glycoproteins secreted from the hyphae chelate Fe II and are small enough in molecular size to penetrate the S_3 layer and produce electron donors such as NADH. Once in the S_2 layer, the glycoproteins catalyse the oxidation of the electron donors, the reaction of which reduces atmospheric oxygen to oxygen species such as $^{\cdot}O_2$ and H_2O_2. The H_2O_2 produced is reduced by Fe II-glycoproteins to $^{\cdot}OH$ and Fe II is oxidised to Fe III. The superoxide reduces Fe III ligated to the glycoproteins, to Fe II which in turn reduces H_2O_2 to form $^{\cdot}OH$'s. The $^{\cdot}O_2$, H_2O_2 and $^{\cdot}OH$ produced in this manner split the cellulose by oxidative reactions with the OH groups present on the pyranose rings of the glucose

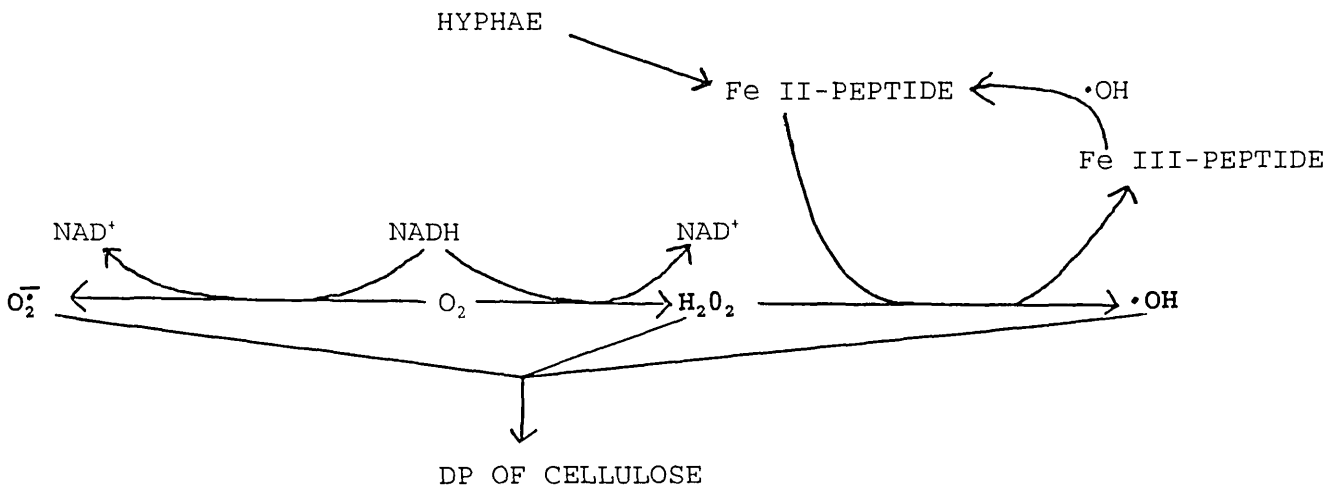

FIGURE 1.3. PROPOSED MECHANISM FOR THE GENERATION OF ACTIVATED OXYGEN SPECIES BY GLYCOPEPTIDES FOR THE CONSEQUENT DP OF CELLULOSE.

monomers. Subsequent oxidation cleaves the pyranose ring resulting in DP (Enoki *et al.*, 1990, 1991; Montgomery, 1982).

1.5.1.3. ACID EROSION

C. puteana is also known to produce acids such as citric, oxalic and acetic acids when it attacks wood lowering the pH of the wood to the region of pH 2-3 (Birkinshaw *et al.*, 1940). The action of acids has been postulated to modify the structure of lignin by hydrolysis allowing access of the enzymes to the partially exposed cellulose and hemicellulose thus permitting degradation to occur (Birkinshaw *et al.*, 1940 and Evans *et al.*, 1989). All wood decay fungi reduce the pH of wood by the production of acids. If the substrate however is too acidic fungal growth is terminated, therefore inbuilt mechanisms to neutralise the acids produced are constantly in action. For example, *C. puteana* produces a larger variety of acids than other wood decay fungi and it has the ability to maintain a constant pH due to the buffering effect of the different types of organic acids liberated (Bech-Anderson, 1987, 1992). In addition, the oxalic acid which is produced, can be neutralised by crystallisation with calcium present in substrates such as concrete, clay or glass (Bech-Anderson, 1987), though unlike *S. lacrymans* a calcium substrate is not a necessity for growth.

All three mechanisms of decay by *C. puteana* described are possible. How and where they interrelate in the decay process is still uncertain, but the quest for a solution to the action of decay fungi on wood continues.

1.5.2. THE EFFECT OF PRESERVATIVES ON *C. PUTEANA*

C. puteana is a standard organism used in the toxicity testing of new preservatives (EN 113, 1982). Preservatives which are effective in the termination of the growth of *C. puteana* include Copper-chrome arsenic (CCA), creosote, tri-butyl tin oxide (TBTO), tri-n-butyl tin naphthenate (TnBTN) and boron formulations. Chemicals such as CCA, TBTO and TnBTN are rapidly being replaced due to their high degree of toxicity to humans and animals. Diffusible biocides such as boron complexes are fungicidal, insecticidal and have low mammalian toxicity and as a consequence are becoming more widely utilised by preservation companies. The knowledge of the toxic limits of fungi to preservatives is important, for example, Viitanen (1991) reported in an analysis of insulating materials that mineral wool (which contains boron) did not prevent the growth of *C. puteana*, in fact in some cases the growth of the organism was stimulated. *C. puteana* is however sensitive to boron when it is in the form of a diffusible salt, for example, boron rods containing boric acid at a concentration of 1.5kg.m^{-3} (Blow and Summers, 1985).

1.6. IMPORTANCE OF EARLY IDENTIFICATION OF DECAY

The identification of fungi which infect structural timbers such as those of maritime artifacts or of buildings, is essential. The early identification of fungi allows treatment regimes with appropriate preservatives to be developed and applied, and more importantly, detection systems for the specific colonisers can be instigated to detect incipient decay.

1.7. METHODS OF IDENTIFICATION.

Until recently identification of microorganisms was by the study of morphological characteristics either at the macroscopic or microscopic levels or by serological tests. Mycologists studying wood decay still rely upon the morphological criteria of various fungal structures, such as conidia for identification (Strom, 1986). Microscopic observations of mycelia and strand structures (Nobles, 1965 and Stalpers, 1978), are also utilised for the identity of wood destroying fungi and classification keys such as those of Bravery (Bravery, 1987) have been developed.

Problems associated with the identification of microorganisms (detailed in chapters 3 and 6) were facilitated with the advent of molecular techniques allowing the analysis of proteins, nucleic acids and antigens. The electrophoresis of such molecules can be used as a basis for the identification and classification of microorganisms. In the case of protein analysis, the process of identification was improved further with the introduction of sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) which provides additional resolving power. SDS-PAGE involves the electrophoretic separation of soluble proteins on the basis of their molecular weight. To visualise the proteins in the gel a number of staining protocols have been used, for example Coomassie blue staining (Meyer and Lambert, 1965) and Silver staining (Switzer *et al.*, 1979).

There is a great diversity in the microorganisms which have been studied using SDS-PAGE. For example, the identification and the grouping of *Clostridium botulinum* by the numerical analysis of their electrophoretic patterns has been studied by Bom *et al.*, (1986). SDS-PAGE is the only technique known that allows the recognition of individual strains of *C. botulinum* by their unique protein profiles. *Thermoactinomyces* strains and *Mycobacterium* species have been identified by SDS-PAGE (Ylonen *et al.*, 1989 and Dejong *et al.*, 1991), in addition to bacterial strains from activated sludge (Hantula *et al.*, 1991). Further, the classification of parasitic organisms of fish eyes' such as metacercariae of the genus *Diplostomum* has also been possible (Faulkner, 1989).

Analysis of fungal isolates using the electrophoretic patterns of soluble proteins extracted from mycelia has been examined. SDS-PAGE has been utilised for the study of *Verticillium* providing information on the taxonomy of the species (Milton *et al.*, 1971). It has allowed the differentiation between strains of yeast on the basis of their secreted proteins (Bouix and Leveau, 1983) and identification of aggressive and non-aggressive strains of *Ceratocystis ulmi* (Jeng and Hubbes, 1983). SDS-PAGE has also allowed the identification of species of *Sclerotina* (Tariq *et al.*, 1985). More recently SDS-PAGE has permitted the identification of different species of *Phytophthora* (Hansen *et al.*, 1988), and *Gaeumannomyces graminis* and *Phialophora* species have also been classified (Mass *et al.*, 1990). The ability to distinguish between strains of *Heterobasidion annosum* and the discrimination of this

organism from other forest pathogens has also been possible (Galbraith, PhD. thesis in preparation).

The application of SDS-PAGE to fungi that are pathogenic to man has also been described. The yeast *C. albicans* has recently been classified by Lee *et al.*, (1986), and other oral yeasts such as *Torulopsis glabrata*, *Yarrowia lipolytica* and *Saccharomyces cerevisiae* have been identified (Maiden and Tanner, 1991).

Initial studies have suggested that fungal identifications have been made which allow the classification of basidiomycetes associated with building timber. *P. placenta* and *Coriolus versicolor* (Kim *et al.*, 1989), have been distiguished from other basidiomycetes by their unique protein profiles. More recently, Palfreyman *et al.*, (1991a and b), demonstrated that the identification of *S. lacrymans* was feasible by the presence of specific protein profiles. In addition evidence for the identity of *L. lepideus* has been presented (Glancy, 1990).

1.8. IMPORTANCE OF EARLY DETECTION OF DECAY

For the purpose of this study, the term "incipient decay" refers to the early stages of fungal colonisation and decay of wood occurring prior to strength loss and/or structural degradation within the wood. The detection of incipient decay in any wooden structure is required in order to locate and identify the colonising organism and further to allow the application of appropriate preservatives prior to the structural damage of the wood. The importance of early detection is outlined below;

a. the wood may be of historical interest, for example the timbers of the *Unicorn* Substantial decay of such timbers would reduce their historical value,

b. the timber in use may be required for strength such as the structural timbers in buildings and electricity poles, or

c. the timber may be impossible to replace in terms of its' location, such as timbers on the *Discovery*.

A combination of a reliable detection system for incipient decay and an effective preservative regime would prevent further degradation and permit the extension of the useful life of in service timber.

1.9. METHODS OF DETECTING FUNGAL DECAY IN WOOD.

In recent years a number of sophisticated methods for the detection of decayed wood have been proposed and will be described in this section. In practice, however, there are two widely used methods (Hayes, 1986). The first is simple hammer sounding, in which the timber is rapped with a hammer, a hollow sound indicating the presence of a decay pocket caused by an organism producing a cavity in the wood cell wall. The second is by the insertion of a sharp blade into the wood. If insertion is possible then evidence that the structure of the timber has been compromised due to an attack by a wood decay organism, becomes available. Neither method described can detect incipient decay.

The methods described for detecting fungal degradation of wood rely therefore on the advanced decay caused by the organisms in the timber. This means that when detection is possible the timber has already been substantially damaged and the only adequate treatment is the wholesale removal and replacement of the decayed wood.

In recent years several quantitative and semi-quantitative methods for the early detection of timber decay have been developed and are discussed in the following sections. Experimental measurement of decay is often recorded in terms of percentage weight loss (expressed as a percentage of the original dry weight of wood). Weight losses of less than 3% are considered to be insignificant since this can simply be due to loss of soluble wood components. Weight losses of greater than 3% therefore are indicative of the biodeterioration of wood (King, 1981). Methods described for the early detection of decay can be divided into two areas; physical and chemical techniques. Below is an evaluation of the applicability of the techniques to the early detection of fungal organisms in any wooden construction.

1.9.1. PHYSICAL TECHNIQUES.

The types of physical properties which have been studied are; conductivity, stability, reflection/absorbance of light, ultrasonics and ion mobility, and are listed below.

a. An estimated value of the extent of decay can be obtained using an electrical conductivity meter

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a. An estimated value of the extent of decay can be obtained using an electrical conductivity meter

by an alteration from a reference measurement of the resistance between two contact points on a twisted probe. This procedure however can produce false positives leading to inaccurate results and the time consuming microbial analysis of all positives is required (Hayes, 1986).

b. The detection of surface degradation by the pilodyn is possible. This is a blunt pin which is fired into the wood by a spring loaded to a constant energy. The strength of the wood is measured by the resistance of the pin as it comes into contact with the surface of the wood (Friss-Hansen, 1980).

The above methods are the two most commonly used physical techniques. A variety of other methods have been proposed and are as follows;

a. Differential Scanning Calorimetry by Baldwin and Streisel (1985), measures extractive free wood and can detect incipient decay up to 5% weight loss, but cannot measure whole wood.

b. X-ray detects cracks and cavities but not incipient decay (Gardner *et al.*, 1980),

c. Modified Computed Axialtomography (CAT scan) (Taylor *et al.*, 1980) is useful for the measurement of decay in electricity poles by producing cross-sectional pictures of knots, growth rings, surface cracks, penetration of

preservatives, and early and severe decay.

d. The application of soft-X-ray microdensitometry to density decreases by wood decay have proved successful in detecting incipient decay. However, it requires much sample preparation and detection of internal decay of large dimensional timbers is doubtful (Yamamoto and Fujii, 1987).

e. Ultrasonic pulse velocity applications can detect advanced decay, and in some cases incipient decay, by an increased measurement from that of sound wood. However, lack of change of measurements from sound wood cannot conclusively indicate that the wood is not compromised by decay fungi. Further research to investigate this problem is required. (Wilcox, 1988).

1.9.2. CHEMICAL TECHNIQUES.

The types of chemical techniques which have been studied include the detection of acids, enzymes and glycolipids. In addition, chemical analysis resulting in the use of spectrophotometry and fluorescence microscopy for the determination of results have been utilised. Details of some applications are given below.

a. Extraction of soluble components of wood/fungus with water and analysis by Infra-Red Spectrophotometry (Gibson *et al.*, 1985 and Nicholas and Schultz, 1986). This method was

successful in detecting incipient decay, but requires large samples for extraction and the use of sophisticated technology renders it unsuitable as a routine testing systems.

b. Ion Mobility Spectrometry (IMS) is based on the thermal release of vapours from wood samples and the subsequent detection of specific chemical moieties in wood by the presence of a specific IMS peak. This peak is only present when the wood is colonised by decay fungi and the chemical nature is unknown. No correlation between the presence of the IMS peak and amplitude, to percentage weight loss has yet been analysed. (Lawrence and Nilsson, 1991).

c. The detection of released acids from fungal hyphae using the colour indicator methyl orange is possible, since fungi reduce the pH of wood prior to substantial decay. This technique has however been shown to have only limited use in the detection of incipient decay (Eslyn, 1979). Similarly, the detection of the presence of basidiomycetes by bromocresol green has been described (Willeitner and Peak, 1979).

d. Indicators to detect enzymes released from fungal hyphae during growth. These indicators allow the detection of the organism and allow the differentiation between the presence of white rot or brown rot basidiomycetes. The

technique is promising, but relies on the production of specific enzymes which are not produced to the same extent by different fungi (Niku-Paavola *et al.*, 1990).

e. Fluorescent microscopy using acridine orange indicates the presence of brown rot organisms at an early stage of decay, but the technique is not applicable to all wood/fungal types (Krahmer, 1982). In addition, the use of plant lectins by Morrell *et al.*, (1986) to locate fungal chitin present in the cell wall of hyphae has been successful in detecting brown, white, and to a limited extent, soft rot fungi in wood.

1.9.3. ORGANISM DETECTION.

The physical changes associated with decay rely on alterations of the gross structure of the wood which are often directly due to advanced decay. In addition detection of all dimensions of timber using these techniques is not always possible. On the other hand, Chemical changes are dependant on the detection of released enzymes, acids, and other compounds from fungal hyphae, or the detection of specific chemicals within the hyphae. These methods do allow the detection of early decay, but due to the nature of the techniques the type of organism responsible for the decay cannot always be determined. A possible alternative to physical and chemical techniques is biological methods. These methods involve the ability to detect the presence or

activity of the organism in wood, rather than changes in the wood itself, or the products released from fungi during the decay process. For instance, catalase activity detection is correlated with the degree of decay, at least, in the early stages of microbial attack (Line, 1981, 1982). However, the sample preparation involved is complex and time consuming. Other examples include the induction of colour changes by the addition of small amounts of specific chemicals to wood samples which indicate the presence of fungi. Details of these techniques are listed in Table 1.1.

More recently the use of immunoassay techniques to detect fungal decay in wood has been initiated. These assays involve the specific interaction of an antigen with an antibody, providing information on the concentration of antigen or antibody present. The method of immunoassay is highly adaptable and is easily modified to suit specific purposes.

1.10. IMMUNOASSAYS: IDENTIFICATION AND DETECTION OF MICROBES

In 1960, Yalow and Berson applied anti-insulin antibodies to the measurement of the hormone insulin in plasma using radio-immunoassay (RIA). It was with this application that the potential of immunoassay, as it is understood today, was discovered due to the specificity and sensitivity of antibodies for the detection of antigens. During the 1970's there was an explosive growth in the application of immunoassays to other biomedical disciplines as well as to industries which previously had little or no contact with immunology (Marks, 1985).

TYPE OF TEST	DETAILS OF TEST	COLOUR CHANGE	REFERENCE
Nobles test:extracellular oxidase	Add a drop of 0.5g gum guaiac-30ml 96% EtOH to colony	Blue colour develops	Nobles (1965)
Laccase (partial enzyme of phenoloxidase)	Add a drop of 0.1M guaiacol-96% EtOH to colony	Purplish after 4-24h	Molitoris;Boidin (1978; (1951)
Cytochrome oxidase	Add a drop of 30mg tetra-methyl-*p*-phenylene-diamine dihydrogen-chloride + 10ml ascorbic acid solution, (15ppm) to colony	Blue discolouration in 2h	Stalpers (1978)
Esterase (hydrolysis of esters)	Add a drop of 1% aq -naphthol acetate: 1% acetone/fast red ITR base (1:1) to colony	Purplish after 4-72h	Stalpers (1978)

TABLE 1.1. EXAMPLES OF ORGANISM DETECTION

Immunoassays provide sensitivity, detecting specific products at very low concentrations, potentially picomolar amounts; specificity in distinguishing between antigens whether simple molecules or whole cells, for example components of blood plasma (Edwards, 1985), and simplicity, since there is no need for lengthy extraction procedures. Immunoassays also have the advantage of a high throughput of samples, speed and most importantly the application to a wide variety of substances. As a result of these attractive features, plus the relatively long life of the reagents, the development of commercial kits was initiated in the 1960's.

The most commonly used immunoassays are the heterogenous assays, in which a separation phase is employed to remove excess reagents which can interfere with the analysis. These assays consist of two procedures, "direct" and "indirect". The "direct" procedure involves the immobilisation of an antigen/antibody on a solid phase followed by reaction with an appropriately labelled antibody/antigen. "Indirect" procedures involve the immobilised antigen/antibody being the target of an unlabelled primary antibody/antigen which is in turn detected by a labelled anti-immunoglobulin probe. The use of two antibodies provides an amplification step which is absent in "direct" procedures and therefore renders the "indirect" procedure more sensitive and thus more widely utilised. A wide range of labels for antibodies have been studied and include radio-isotopes, fluorescence, luminescence and enzymes. The last is more commonly employed due to the economic attraction and also its ease

of use (Voller and Bidwell, 1980). The underlying principle for all immunoassays remain the same although many modifications are available (Engvall, 1980).

The applications of these immunological techniques has been widespread in many areas of biology. For example in the discipline of virology, the antigenic structure of the influenza virus hemagglutinins has been studied using EIA in an attempt to elucidate the antigenic variation of the virus (Skehel and Wiley, 1985). In bacteriology, various organisms have been identified by the use of immunotechnological methods. For example, *Spiroplasma* antigens have been identified by precipitin arcs using immunoelectrophoresis (IE) (Archer and Townsend, (1981). A study of *Mycobacterium paratuberculosis* and other *mycobacterin* dependant *mycobacteria* using immunodiffusion (ID) indicated, by the presence of specific precipitin bands that these organisms were variants of *M. avium* (McIntyre and Stanford, 1986) and identification studies of *Salmonellae* (Brown and Hormaeches, 1989) and *Thermoactinomyces* (which causes Farmer's Lung Disease) have been highlighted (Ylonen *et al.*, 1989). In addition, identification of specific bacteria in plaque samples and on roots of extracted teeth has been possible by the detection of antigens using scanning electron microscopy in conjunction with immuno-gold labelling (Carrass *et al.*, 1990).

The use of immunological methods in mycological research is limited compared to their widespread use in the fields of bacteriology and virology. The limitation of fungal analysis by such techniques may be a direct consequence of

the complexity of the antigenic nature of fungal organisms and similarly due to the complicated lifecycles of some fungi. In addition, initial studies of fungi only utilised whole organisms as immunogens, resulting in antisera with low specificity. Further, the ability of fungal organisms to morphologically and physiologically alter to various environmental conditions contributes to their complexity (Longbottom and Pepys, 1979). For example, *Sporotrichosis* can cause cutaneous and subcutaneous infections and can also alter its morphology to enable it to survive in lungs, bones and the meninges (de Albornoz *at al.*, 1984). A greater understanding of fungal growth and development in recent years and the investigations of the use of different types of fungal extracts as immunogens, has allowed a proliferation of the use of immunological assays. Such assays are now routinely used in medical and plant mycology.

1.10.1. MEDICAL MYCOLOGY.

Medical mycology has witnessed an increased use of immunological techniques in the last 20 years. ID, first introduced by Ouchterlony in 1958 and crossed-IE techniques (Grabar and Williams, 1953) were adopted as methods for the immunoidentification of fungal species such as *Histoplasma*, *Aspergillus* and *Penicillium* by the recognition of specific antigens (Kaufman *et al.*, 1983). EIA and immunoblotting have been extensively used to characterise antigens, for example, antigens of the organism *Plasmodium falciparum* (Chumpitazi *et al.*, 1987). Immunofluorescence (IF) and immunoblotting have been described in the analysis of antigens of *P. falciparum* (James *et al.*, 1987) and in the

grouping of strains of *Aspergillus fumigatus* by the recognition of unique antigen profiles (Burnie *et al.*, 1989). ID, EIA and immunoblotting have together allowed the detection and analysis of antibodies to *A. fumigatus* (Brouwer, 1988). EIA has also been described for the detection of anti- *A. fumigatus* IgG (Shale and Faux, 1985).

Many of these investigations involved "exoantigens", which have been described by Kaufman and Standard in 1987 as

> "antigens or soluble immunogenic macromolecules produced by fungi early in their development".

The introduction of exoantigens has to some extent simplified the problems of specificity, which hampers the use of immunological reagents in mycology. These highly specific antigens are readily detected in culture broths or aqueous extracts of slant cultures. The diagnosis of disease was achieved by carrying out exoantigens tests. These test involved the interaction between exoantigens and the antibodies that were generated to precipitate them. The precipitates are checked with reference precipitates in ID to establish the identity of the organism. The use of such identification of antigens has allowed the immunodiagnosis and detection of dimorphic pathogenic fungi such as *Blastomyces dermatitidis* (Sekhon *et al.*, 1986), *Coccidiodes immitis* (Cox and Britt, 1986), *Histoplasma capsulatum* (Kaufman *et al.*, 1983) and *Paracoccidioides brasiliensis* (Kaufman, 1987). However, there are limitations to the exoantigen technique and these will be discussed in chapter 3.

1.10.2. PLANT MYCOLOGY.

The use of immunological methods for the identification and detection of fungal diseases in plants has been in evidence for a number of years. For example, the classical immunoprecipitation methods of ID and IE have been used to antigenically differentiate between species of *Ceratocystis* (Amos and Burrell, 1967) and to study the antigenic relationship between species within the genus *Smittium* (Sangar *et al.*, 1972).

More recently, however, the ability of EIA to detect plant pathogens occurring at much lower concentrations than is possible with the formerly used classical immunoprecipitation methods has been highlighted (Clark, 1981). EIA has also allowed the development of a monitoring system for the detection of the spread and interaction of endomycorrhizal fungi with each other and the resident fungal flora (Rice *et al.*, 1984). Further, the detection of *Phoma exigua* in infected potato tissue by EIA (Aguelon and Dunez, 1984) has also been made possible. Alternatively, the potential of methods such as IF have been outlined for the detection of fungal mycelium in barley grains (Warnock, 1971) and the study of *Botrytis cinerea* isolated from rotting strawberries (Preece and Cooper, 1969). Further, electron microscopy using immuno-gold labelling has allowed the visualisation of the plant pathogen *Colletrichum lindemuthianum* and the study of the distribution of plant pathogenic products within infected tissues (O'Connell *et al.*, 1986).

1.10.3. WOOD DECAY

As a direct consequence of the success of immunological techniques for identifying and detecting plant and human fungi, such methods have been applied to the field of wood decay. The analysis of fungi associated with wood decay, by immunological methods has had a relatively slow growth, but in recent years, the use of such techniques has increased. For example, ID and IE have been used to identify *Fusarium* species (Hornock, 1980) and *Gloeophyllum* species (Madhosingh and Ginns, 1975). Immunoblotting has recently been reported for the identification of *S. lacrymans* (Vigrow *et al.*, 1991) and Goodell and Jellison (1986) have developed an EIA system to detect *P. placenta* using polyclonal antisera. Visualisation of this organism by IF microscopy has also been reported (Goodell *et al.*, 1988). Further, Palfreyman *et al.*, (1987, 1988a and b), have studied the wood decay fungi *C. versicolor* and *S. lacrymans* using immunoblotting and immuno-cytochemical techniques and observed unique antigenic profiles for both fungi which have allowed the detection of both fungi using these systems. The estimation of fungal colonisation of wood, by EIA, has been reported by Breuil *et al.*, (1987, 1988) using polyclonal antisera to the sap-stain organism *Ophiostoma*. Further studies by Glancy *et al.*, (1989) and Palfreyman *et al.*,(1988a), on the wood decay organism *Lentinus lepideus*, include ID, dot-immunoblotting and western blotting techniques for the antigenic identification and detection of the organism. Similarly, immunoassays coupled to microscopy have widened the area of research with studies of the hyphal sheath of *P. placenta* using immunoscanning electron microscopy (Green *et al.*, 1991), and the

immunolocalisation of extracellular fungal metabolites from *Tyromyces palustris* by Kim (1991) using immunogold transmission electron microscopy.

These studies have indicated that it is possible to identify and detect fungi, particularly within natural substrates. Such detection has also been confirmed using other fungi (Benhamou *et al.*, 1986; Dewey and Brasier, 1988). The detection of individual types of organisms by immunoassay techniques using polyclonal antisera, is not always possible due to the high cross-reactivity which is observed for many antisera. Thus the production of highly specific antibody probes is often desirable.

1.10.4. HYBRIDOMA TECHNOLOGY

The hybridoma technique involves the production of monoclonal antibodies (MAbs). These antibodies are highly specific for a particular antigen and are produced by the fusion of specific antibody forming cells with tumour cells which can grow indefinitely (Kohler and Milstein, 1975). The main advantages which monoclonal antibodies have over polyconal antibodies are in the defined specificity, homogeneity and the ability to obtain practically unlimited quantities of the same antibody in a reproducible manner. Possibly the most exciting advantage of hybridoma technology is the generation of monoclonal antibodies to almost any epitope, provided that specific assay procedures can be developed for the required purpose (Eshhar, 1985).

Since the introduction of MAbs in 1975, there has been exponential growth in the literature relating to MAb

production and utilisation, and numerous uses of MAbs particularly in the medical field are now apparent. For example, the application of MAbs to the study of viruses such as influenza, rabies, poliovirus and hepatitis have provided much information on the antigenicity of viral proteins, virus-host interactions and the serological characterisation of viral isolates (Gerhard and Bachi, 1986). The high specificity of MAbs has also led to the accurate identification of similar strains of virus, such as *Herpes simplex* Types I and II (Campbell, 1984). In addition, the application of MAbs to the study of bacterial organisms has allowed an insight into the structure of particular organisms, e.g. the determination of the structure of the toxin of *Vibrio cholerae*, in particular the subunit responsible for toxicity (Robb *et al.*, 1982). Further, purified proteins and whole cells of *Neisseria gonorrhoeae* have been used to produce MAbs to aid in the serological classification of the organism (Tam *et al.*, 1982) and the demonstration of a surface antigen of *Clostridium tyrobutyricum* by use of immunoblotting with MAbs has allowed immunological characterisation of this bacterium (Gueguen *et al.*, 1990). MAbs are also paramount in the detection and therapy of various pathological conditions, including malignant disease, e.g. colon, ovarian and breast cancers (Byers and Baldwin, 1988).

The analysis of organisms causing fungal infections by the application of MAbs has also been possible. For example, *Microsporum canis* and *Histoplasma capsulatum*, previously identified by the exoantigen technique, have also been identified by the employment of MAbs produced against specific exoantigens (Polonelli and Morace, 1985; Hamilton

et al., 1990). Similarly strains of the yeast *Candida albicans* (which proliferates when the immune system is compromised), have been serologically identified by the presence of specific antigenic determinants detected by a MAb (Polonelli and Morace, 1986). In addition, fungal organisms which cause the destruction of plant tissue have been analysed by the application of MAbs. For example, Dewey *et al* (1989) produced a specific monoclonal antibody to the components of surface washings of *Humicola lanuginosa* which infects rice grains. This monoclonal antibody was used to develop a highly sensitive "DIP-STICK" immunoassay to detect the organism in infected rice grains.

MAbs therefore provide a means of specifically detecting, identifying and characterising antigens from a range of organisms, which provides physiological, metabolic and phenotypic information. This information may subsequently be utilised to facilitate the task of diagnosing and/or eliminating infection of all types of biological material, including wood.

1.11. DESCRIPTION AND AIMS OF PROJECT.

This project was initiated to develop and apply molecular and immunological techniques to the identification and detection of *C. puteana*. This organism was chosen for study since it is considered to be the wet rot organism which causes most damage to building timbers in the United Kingdom. In addition, evidence of outbreaks of wet rot due to *C. puteana* were present in the maritime artifacts under study. The systems developed however are applicable to other decay fungi in any wood environment.

The project includes the investigation of four particular areas:

a. The development of identification systems for wet rot fungi, specifically *C. puteana*.

b. The molecular and immunological analysis of *C. puteana*.

c. Specific detection systems for *C. puteana*.

d. The application of systems developed to the identification and detection of organisms resident in maritime artifacts.

CHAPTER 2

MATERIALS AND METHODS

2.1. FUNGAL ISOLATES

The majority of fungal isolates used in this study were obtained from the culture collection at the Forest Products Research Laboratory (FPRL), Building Research Establishment, Garston, U.K. Alternatively fungi were obtained from;

a) Bundesanstalt fur Materialprufung, Berlin-Dahlem, Germany (BAM);
b) Forest Products Laboratory, Madison, Wisconsin, U.S.A. (MAD);
c) CAB International Mycological Institute, Kew, U. K. (CMI/IMI);
d) Centraalbureau voor Schimmelcultures, Baarn, The Netherlands (CBS);
e) Professor T. Nilsson, Swedish University of Agricultural Sciences, Uppsala, Sweden (BM),
f) Northern Research Station of the Forestry Commission, Midlothian, UK (NRS).

Fungi were also isolated during the project and are referred to as DIT (Dundee Institute of Technology) isolates. Table 2.1. provides details of all fungi used.

2.2. CULTURE OF ORGANISMS

All fungi were cultured at 22°C in the dark on 2% (w/v) purified agar (A; Oxoid No. L28)/5% (w/v) malt extract (MX; Oxoid No. L39). Stock cultures of fungal isolates were inoculated onto 2% A/5% MX slopes, incubated at 22°C for 7-10 days then stored at 4°C. Stock cultures were subcultured every 6 months.

BROWN ROT FUNGI

Coniophora puteana (Schumacher ex Fr) Karsten FPRL 11E
Coniophora puteana (Schumacher ex Fr) Karsten FPRL 11A
Coniophora puteana (Schumacher ex Fr) Karsten FPRL 11B
Coniophora puteana (Schumacher ex Fr) Karsten FPRL 11Q
Coniophora puteana (Schumacher ex Fr) Karsten BAM 15
Coniophora arida (Fr) Karston FPRL 411
Coniophora marmorata Desm FPRL 410
Serpula lacrymans (Schumacher ex Fr) Gray FPRL 12C
Poria placenta (Fr) Cooke Sensu J. Eriksson FPRL 280
Fibroporia vaillantii (DC ex Fr) Parm FPRL 14H
Amyloporia xantha (Fr) Bondartsev & Singer ex Singer FPRL 62F
Laetiporus sulphureus (Buller ex Fr) Murrill FPRL 29
Gloeophyllum trabeum (Pers ex Fr) Murrill BAM 109
Gloeophyllum sepiarium (Wulfen ex Fr) Karsten FPRL 10D
Neolentinus (Lentinus) lepideus (Fr ex Fr) Fr FPRL 7F
Daedalea quercina (L ex Fr) FPRL 38
Peniophora gigantea (Fr ex Fr) Massee FPRL 175B
Leucogyrophana (Serpula) pinastri (Fr) Cooke FPRL 141B
Paxillus panuoides (Fr ex Fr) Fr FPRL 8B
Poria incrassata (Berk & Curtis) Burt FPRL 71

WHITE ROT FUNGI

Pleurotus ostreatus (Jacq ex Fr) Kummer FPRL 40A
Schizophyllum commune (Fr) FPRL 9
Daldinia concentrica (Bolt ex Fr) Ces & de Not FPRL 26E
Stereum sanguinolentum (Alb & Schwein ex Fr) Fr FPRL 27D
Coriolus versicolor (L ex Fr) Quelet MAD 697
Ceratocystis picea
Heterobasidion annosum Fr (Bref) FPRL 41E

TABLE 2.1.

Verticillium spp. NRS 69.0
Hyphoderma puberum (Fr:Fr) Wallr. CBS 464.86
Verticillium lecanii (Zimm.) Viegas CBS 546.81
Verticillium cf. lamellicola CBS 912.70A
Phellinus cf. igniarius CBS 349.74

NON-BASIDIOMYCETES

Trichoderma harzianium IMI 206040
Paeciliomyces varoitti DIT pi
Cladosporium resinae BM 13385-1-22A
Trichoderma polysporium IMI 206039

DIT-ISOLATES (from fruiting body, wood, strand and mycelial samples)

U1
U2
U3
U4
U51
U52
U61
U62
U9
U12
U12b
U13
U17
U17b
U20
D3
D3f
DIc
Ua
Ub
Uc
Ud
Ue
Ug
Ui
Uk
Um

TABLE 2.1 CONTINUED

UNICORN CORE ISOLATES (Ci)

UCi 1a
UCi 10b
UCi 13
UCi 24
UCi 27

TABLE 2.1. LIST OF FUNGAL ORGANISMS USED IN THIS STUDY

2.3. PRODUCTION OF FUNGAL MYCELIUM FOR THE PREPARATION OF PROTEIN EXTRACTS

Throughout this project, *C. puteana* FPRL 11E was used as a standard organism. Three extracts of this organism were used viz., a whole cell mycelial extract (WM); an exoprotein extract (EP) and a growing tip extract (GT). Additional extracts from distinct morphological regions of *C. puteana* fungal mycelia including whole cell and exoprotein preparations of these extracts were used. Details of their preparation are discussed in sections 2.3.3. and 2.3.4.

2.3.1. WM EXTRACTS

WM extracts were prepared from fungi grown on 5% (w/v) malt extract broth (MXB; Oxoid No. CM57) plates. The MXB plates were incubated at 22°C in the dark until approximately 75% of the surface of the broth was covered by mycelia (10-14 days). Mycelial mats were harvested without the original cores and washed on Whatman No. 1 filter paper (Whatman International Ltd.) with distilled water until the filtrate ran clear. The mycelia were stored at -20°C until required.

2.3.2. DEVELOPMENT OF METHOD FOR EXOPROTEIN PRODUCTION

Four exoprotein extracts were prepared (EP1-4).

2.3.2.1. EXOPROTEIN 1 (EP1)

Screw-capped test tubes containing 8ml slopes of 2% A/5% MX were inoculated with 5mm diameter cores of stock fungi

grown on plates of 2% A/5% MX (w/v). These slope cultures were incubated for 14 days at 22°C in the dark and then overlayed with 10ml distilled water at 22°C for 24h. The resultant s/n was centrifuged at 13,000 rpm (Microcentaur, MSE Scientific Instruments) for 10 min, membrane filtered (0.2µm millipore membrane, Millipore Ltd.), dialysed through dialysis membrane and stored at -20°C.

2.3.2.2. EXOPROTEIN 2 (EP2)

Cultures were incubated on 2% A/5% MX (w/v) slopes at 22°C for 14 days in the dark then overlayed with 10ml distilled water. After a further incubation of 1h at 22°C, the resultant s/n was centrifuged at 13,000 rpm for 10 min and membrane filtered (0.2µm millipore membrane). The filtrate was stored at -20°C.

2.3.2.3. EXOPROTEIN 3 (EP3)

Fungal mycelia were grown on 5% MXB (w/v) at 22°C for 14 days in the dark and washed on Whatman No. 1 filter paper as described in 2.3.1. The resultant filtrate was retained and stored at -20°C until required.

2.3.2.4. EXOPROTEIN 4 (EP4)

Fungal mycelia were cultured on 2% A/5% MX slopes at 22°C in the dark for 5-15 days. The mycelia were overlayed with 1ml phosphate buffered saline (PBS; 10mM, pH 7.4; Appendix 1A) and brought into contact with the buffer by gentle stroking of the hydrophobic surface of the mycelia with a spatula. The buffer-soluble surface washings (SW) were

extracted after gentle agitation of the tube. The extract was centrifuged at 13,000 rpm for 10 min and the s/n stored at -20°C. To produce a greater quantity of exoproteins, fungi were cultured on 2% A/5% MX in 9cm petri-dishes, overlayed with 3ml PBS and further prepared as described above.

2.3.3. GROWING TIP (GT) AND ADDITIONAL PROTEIN EXTRACTS

Three morphologically different regions of the fungal mycelium, the growing tip (GT), intermediate mycelia and aged mycelia (Figure 4.5.) were excised using a sharp blade ensuring the exclusion of agar. An alternative GT extract was also prepared by excising 0.5mm of the outer edge of the advancing hyphal front according to the method of Vigrow (PhD. thesis, 1992). Samples required for whole mycelial protein extracts were stored at -20°C until required. Samples required for exoprotein production were shaken o/n at 4°C with 1ml PBS, centrifuged at 13,000 rpm for 10 min and the resultant s/n stored at -20°C.

2.4. LYOPHILISATION OF FUNGI

For long term storage extracts were lyophilised in round bottomed flasks (large samples) or in pierced eppendorf tubes (small samples) using a vacuum freeze drier (Model No. FD 500/60, Birchover Instruments Ltd., or Freeze Drier Micro Modulyo, Edwards, High Vacuum International). Lyophilised samples were collected into cryotubes (Nalgene Cryoware, Nalge Company) and stored at -180°C. Any remaining lyophilised material was stored in universal bottles at -20°C.

Exoprotein extracts prepared as described in section 2.3.2. were collected into pierced eppendorf tubes and de-oxygenated by a slow vacuum prior to lyophilisation. Samples were lyophilised o/n and stored in cryotubes at -180°C.

2.5. THE PRODUCTION OF ANTISERA

All antisera were produced against *C. puteana* FPRL 11E in either New Zealand White rabbits (standard antisera) or in Wistar rats.

2.5.1. ANTISERUM TO WM EXTRACTS

2mg of lyophilised WM mycelia were ground up in 1ml PBS to produce a fine slurry. 200µl aliquots of this slurry were emulsified in 1ml Freund's Complete Adjuvant (Gibco Laboratories) to give a final concentration of 1:1 and injected subcutaneously (sc) at 6 dorsal sites on a New Zealand White rabbit. Booster injections of the antigen preparation mixed with Freund's Incomplete Adjuvant (1:1; Gibco Laboratories) were given 2 weeks later. A stock of pre-immune control serum was obtained by bleeding the rabbit prior to immunisation. Blood was collected 7 days after the booster injections from the marginal ear vein, allowed to clot at 4°C o/n and the serum separated from the red blood cells by centrifugation at 2,500g (IEC Centra-4B Centrifuge, International Equipment Company) for 10 min. The s/n serum fraction was stored in 100µl aliquots at -20°C and designated by the code 88/1.

2.5.2. ANTISERA TO SW EXTRACTS

Exoprotein preparations (EP) were used as immunogens to produce antibodies in Wistar rats (1 rat per EP extract). A stock of pre-immune control serum was obtained by bleeding the rats prior to immunisation. The production of antisera to EP1 and EP2 was according to the method described in 2.5.1., whilst the production of antisera to EP3 and EP4 was based on the method described by Dewey *et al.*, 1989. The individual methodologies are detailed in Table 2.2. Storage of all antisera was at -20°C.

2.5.3. ANTISERA TO GT EXTRACTS

Antisera were raised in Wistar rats. Antigens were prepared by grinding 2mg lyophilised WM-GT proteins in 1ml PBS. Immunogen preparation and immunisation was as described in section 2.5.1. Serum was collected as previously described and stored at -20°C. The antisera were designated by the codes 90/2 E and F. A further 4 rats were immunised using the same protocol but with a concentration of antigen of 1mg per ml PBS. The antisera produced were coded 90/2 A-D.

2.6. DETERMINATION OF TITRE AND SPECIFICITY OF ANTISERA

The titres of all antisera were assessed using EIA, and specificity was determined by EIA and western blotting (2.7., 2.8. and 2.10.)

SAMPLE	SITE	AMOUNT	ADJUVANT	BLEEDS	ANTISERA
EP1	sc ventral	0.5ml of 90 fold dilution of sample	/	2	88/2
EP2	sc ventral	0.5ml of 0.2mg per ml sample	/	2	88/5
EP3	ip	1ml of 1mg per ml sample	X	4	88/7
EP4	ip	0.6ml of undiluted sample	X	4	88/8*

KEY

/ - Adjuvant used
X - Adjuvant not used
sc - Subcutaneous injection
ip - Intraperitoneal injection
* - Large quantities of this antiserum were produced for subsequent analyses, a further 4 rats were immunised using the same protocol. The blood was collected by exsanguination and serum prepared in the usual manner. The antisera were designated by the codes 88/8 A-D.

TABLE 2.2.

METHODOLOGIES FOR THE PRODUCTION OF ANTISERA TO EXOPROTEIN EXTRACTS

2.7. ENZYME IMMUNOSORBENT ASSAY (EIA)

Antigen preparations were prepared as described in section 2.3. WM antigens were diluted to a concentration of 50µg per ml of PBS after optimisation of EIA assay conditions as described in the following section. SW preparations were diluted 1:20 (v/v) in PBS according to the method of Dewey *et al.*, (1989). Details of solutions marked with an asterisk (*), in this and subsequent sections are given in the relevant section of Appendix 1.

100µl aliquots of the diluted antigens were dispensed into microtitre wells (Titertek immunoassay plate, Flow Laboratories or Nunc Immunomodules, Nunc Incorporated) and incubated at 4°C o/n to allow antigen binding to the wells. The wells were washed using an automated plate washer (Ultrawash II, Dynatech) by flooding 6 times with PBS/0.05% Polyoxyethylenesorbitan monolaurate (Tween 20); (PBS/0.05%tw), after this and all further steps. All incubations were carried out at RT in a moist sandwich box. Non-specific binding of both primary and secondary antibodies to the wells was prevented by blocking free binding sites with 250µl aliquots of PBS/10% Newborn Calf Serum (NCS; Gibco Laboratories)/0.5%tw for 1h. 100µl aliquots of the primary antiserum diluted in PBS/5%NCS/0.05%tw were added and incubated for a further hour. Specific binding was detected by incubation (1h) of 100µl aliquots of horseradish-peroxidase anti-rabbit/rat IgG (HRP-Ab2). HRP-Ab2 for rabbit antibodies (Scottish Antibody Production Unit; SAPU) was diluted 1:500 (v/v) in PBS/5%NCS/0.05%tw and for rat antibodies was diluted 1:250 (v/v) in the same diluent. Enzyme activity was detected using tetra-methyl benzidine (TMB) substrate solution*, the

colour reaction being terminated by the addition of 2M sulphuric acid (BDH) after 30 min. The extent of the reaction was measured spectrophotometrically at 405nm or at 450nm using an automated microtitre plate reader (Titertek Multiskan Plus, Flow Laboratories). The following negative controls were used;

a) pre-immune serum, to give background values of non-specific binding of antiserum components,

b) no antigen, to provide information on non-specific binding of the primary antibodies and,

c) no first antibody, to indicate any non-specific binding of the second antibodies.

2.7.1. CHEQUER-BOARD ASSAY EIA

The chequer-board assay for the optimisation of assay conditions for subsequent titre determinations was based on the protocol described by Cambell (1984). Doubling dilutions of the antigen from an initial concentration of 100µg antigen per ml PBS were prepared using PBS as the diluent. Doubling dilutions of primary antiserum were prepared from an initial dilution of 1:100 (v/v) to a dilution of 1:102400 (v/v) in PBS/0.05%tw. 100µl of the first antigen dilution were applied to the wells of row A of a microtitre plate. This was repeated for rows B-G with the various dilutions of antigen. Row H was filled with 100µl PBS per well. All wells were blocked o/n and washed as previously described. 100µl of the first dilution of antiserum were applied to the wells of column 1 and column 2-11 were filled with the various dilutions of the

antisera. Column 12 was filled with 100µl of PBS per well. The wells were washed after 1h and incubated with HRP-Ab2 for a further hour. After washing the plate, colour development was achieved by the addition of TMB substrate solution, the reaction terminated by sulphuric acid and the absorbance measured at 405nm or 450nm. Full details of the washing procedures and volumes used are described in section 2.7.1. For the purpose of this thesis, titre is used to indicate the lowest antigen concentration at which the highest absorbance value is still observed.

2.8. SODIUM DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Protein preparations were separated on 5%/15% acrylamide SDS-gradient gels (LKB 2001 Vertical Electrophoresis Unit) using the discontinuous buffer system, following the method of Laemmli (1970) as modified by Marsden *et al.*, (1978). WM protein preparations were used at an initial concentration of 6.25mg per ml in PBS. SW preparations were used at an initial concentration of 1ml SW extract, which was subsequently lyophilised and reconstituted in 50µl PBS/boiling mix (2:1; v/v).

2.8.1. PREPARATION OF SAMPLES

WM samples ground in PBS were diluted 2 parts sample:1 part boiling mix* (v/v). WM and SW samples (prepared as described above) were heated to 100°C for 2-5 min (depending on the sample volume) and centrifuged at 13,000 rpm for 10 min. The resultant s/n were used as a source of proteins for electrophoresis.

2.8.2. PREPARATION OF RESOLVING GEL

The preparation of the resolving gel solution is shown in Table 2.3(i). After the addition of ammonium persulphate* and N,N,N,N-Tetramethylethylenediamine (TEMED; Koch-Light Laboratories Ltd.) the gels were poured using a standard gradient maker and a Microtube peristaltic pump. The solutions were allowed to enter the glass gel formers at a constant rate (~3ml per min) through a narrow tube. The solution was overlayed with butan-1-diol to exclude oxygen while the gels polymerised (20 min). An indication of the

completion of polymerisation was the development of a water separation phase between the gel and the butan-1-diol. At this stage the butan-1-diol was decanted, the gel surface washed twice with distilled water and excess water removed by blotting. The resolving gel was overlayed with resolving gel buffer* and stored at RT until required (normally next day). Alternatively, the stacking gel was prepared immediately as detailed below.

(i)RESOLVING GEL

CHEMICALS	SOLUTIONS	
	5%	15%
Acryl/bisacryl (res. gel)*	2.60ml	8.00ml
Resolving gel buffer	4.00ml	4.00ml
Distilled water	9.40ml	1.60ml
Ammonium persulphate (1%)#	100.00µl	43.40µl
TEMED#	6.60µl	6.60µl
Glycerol	---	2.40ml

(ii)STACKING GEL

CHEMICALS	VOLUMES
Acryl/bisacryl (stack. gel)*	2.0ml
Stacking gel buffer	3.0ml
Distilled water	7.0ml
Ammonium persulphate (1%)#	100.0µl
TEMED#	5.0µl

KEY

Acryl/bisacryl - Acrylamide/bisacrylamide

\# - solutions were added immediately prior to pouring gels.

* - acryl/bisacryl resolving and stacking gel solutions, (appendix 1B).

TABLE 2.3.

PREPARATION OF SOLUTIONS FOR THE PRODUCTION OF RESOLVING (i) AND STACKING(ii) GELS

2.8.3. PREPARATION OF STACKING GEL

The preparation of the stacking gel solution was as described in Table 2.3(ii). After the addition of ammonium persulphate and TEMED, the solution was injected onto the washed resolving gel (excess resolving gel buffer was removed by two washes with distilled water) using a syringe. A 20-toothed plastic comb was inserted and the gel was allowed to set at 30°C. Immediately after polymerisation (30 min) the comb was removed, excess stacking gel solution displaced by two rinses with distilled water and the gel formers blotted to extract excess water.

2.8.4. APPLICATION OF SAMPLES AND ELECTROPHORESIS

20µl aliquots of the sample s/n were loaded into separate wells. Molecular weight standards* (SDS-7, Sigma Chemical Company) were always used as controls. Electrophoresis was carried out in tank buffer* at 35mA/gel for 3.5-4h or until the bromophenol blue marker dye was approximately 3mm from the bottom of the gel.

2.9. SILVER STAINING OF SDS-PAGE GELS

After electrophoresis, proteins were visualised using the silver staining protocol of Blum *et al.*, (1987). All volumes used were 250ml and those solutions containing sodium thiosulphate or silver nitrate were freshly prepared in order to obtain sensitive and reproducible staining.

Gels were incubated in a fixing solution (Fix*) for 60 min o/n at 4°C, followed by three 20 min washes in Wash A*. For

1 min the gels were pretreated in a solution of sodium thiosulphate (Pretreat*) followed by three 20 second rinses in distilled water to remove excess sodium thiosulphate. Impregnation in a silver nitrate solution (Impregnate*) for 20 min was followed by two 20 second rinses in distilled water to remove excess chemical. The development of coloured protein bands in a Develop* solution of sodium carbonate/sodium thiosulphate was rapid in most cases but the rate of appearance depended upon the concentration of proteins within the gel. Development was slowed by 2 rinses in distilled water (2 min per rinse) and finally stopped by the addition of an acetic acid/methanol solution (Stop*) for 10 min. Gels were finally washed in Wash B* for 20 min and stored in either polythene bags containing distilled water at RT or by vacuum drying gels (Blum *et al.*, 1987).

Prior to drying, gels were shaken at 4°C for 30 min in 30% methanol followed by a further 30 min in 3% glycerol. Drying was under vacuum at 80°C for 60 min between cellophane. Storage of gels was at RT.

2.10. WESTERN BLOTTING

Western blotting of separated proteins onto Immobilon (Millipore Ltd., U. K.) was achieved by using the Sartoblot semi-dry electrophoretic transfer system (Sartoblot II, Sartorius Ltd.) following the method of Towbin *et al.*, (1979) as modified by Kyhse-Anderson (1984).

Immediately after electrophoresis, gels were incubated in cathode buffer* (pH 9.4) for 5 min at RT. A gel sandwich was constructed on the base of the Sartoblot apparatus (cathode) after both the cathode and the anode (lid) were

rinsed with distilled water at 4°C. Three sheets of Whatman No. 1 chromatography paper (Whatman International Ltd.) soaked in cathode buffer were placed on the cathode base. The gel also soaked in cathode buffer was placed on top of the chromatography paper. Immobilon wetted in analaR methanol (BDH) and soaked in anode 2 buffer* (pH 10.4) was placed on top of the gel and covered with 1 sheet of chromatography paper from anode 1 buffer* (pH 10.4). The transfer of proteins was carried out at 4°C at 0.8mA/cm^2 of gel for 30 min followed by a further 1.2mA/cm^2 of gel for 30 min.

2.10.1. POST-BLOT TREATMENT OF IMMOBILON

Strips of immobilon containing separated standard proteins were removed from the remaining membrane and stained using the method of Hancock and Tsang (1983) to verify efficient transfer of proteins. The strips were rinsed twice in PBS/0.05%tw and incubated in Pelikan ink/PBS (1:1; v/v) for 1h. On removal from the ink the strips were washed twice in PBS and allowed to dry.

The remaining immobilon was washed twice with PBS/0.05%tw and non-specific binding sites were blocked for 1h at 4°C by incubation in 20ml PBS/10%NCS/0.5%tw followed by 6 washes with PBS/0.05%tw. Incubation with 20ml of the appropriate *C. puteana* FPRL 11E antiserum was carried out o/n at 4°C. Antisera were diluted appropriately in PBS/5%NCS/0.05%tw. Following 6 washes with PBS/0.05%tw incubation was at RT using HRP-Ab2, anti-rabbit antiserum (1:500 (v/v)) or anti-rat antiserum (1:250 (v/v)) both diluted in PBS/5%NCS/0.05%tw. Excess enzyme-linked antibody was removed by 6 washes in PBS/0.05%tw followed by 3 washes

in PBS. The detection of bound antibody was with diaminobenzidine enhanced with nickel chloride* (DAB-NiCl; Harlow and Lane, 1988).

2.11. DOT IMMUNOBLOTTING

100µl aliquots of antigen was applied to immobilon membrane (presoaked in analaR methanol) using a dot-blotter (Dot-Blotter II; Biorad). The antigen solutions were allowed to soak into the immobilon over a low vacuum for 2h. The membrane was removed from the dot-blotter and stained according to the antibody staining protocol described in section 2.10.1., using HRP-labelled anti-mouse gamma globulin.

2.12. IDENTIFICATION OF FUNGI

2.12.1. ANALYSIS OF PROTEIN PROFILES

The protein profiles obtained using SDS-PAGE analysis followed by silver staining were interpreted numerically by a Percentage Similarity Index based on that described by Palfreyman *et al.*, (1991a). The index was calculated by counting the number of common protein bands of the test organism compared to the reference (REF) organism i.e.

$$\% \text{ similarity} = \frac{\text{No. common test bands cf REF}}{\text{total no. REF bands}} \times 100$$

The index was only used if 20 protein bands were evident in a sample. The main reference organism used throughout this study was *C. puteana* FPRL 11E.

2.12.2. ANALYSIS OF ANTIGEN PROFILES

Antigen analysis of blotted membranes was by reference to the number of antigens always evident when *C. puteana* FPRL 11E was probed with antiserum (88/1). Comparison of other antigens of fungal organisms was by scoring their antigens for the presence of the 5 major antigens of *C. puteana*.

2.12.3. MORPHOLOGICAL IDENTIFICATION

Samples were studied for identification purposes by analysis of the environmental circumstances in which they were found and by morphological analysis after initial observation and/or isolation. Both studies were based on information from literature on wood decay fungi (Cartwright and Findlay, 1958; Coggins, 1980 and Bravery *et al.*, 1987).

2.12.4. ISOLATION PROCEDURES

2.12.4.1. ISOLATION FROM FRUITING BODIES

Fruiting bodies were surface washed in 70% ethanol, cored using a 5mm corer, cultured on 2% A/5% MX containing benomyl and streptomycin (Appendix 1E) and the mycelia allowed to become established. Once a pure culture was established the mycelia were analysed by microscopy for clamp connections to determine the presence of basidiomycete mycelia. Clamp connections were visualised by the clear "Sellotape" staining method described in section 2.12.4.4. Growth rates of isolates were recorded by measuring the radial growth of the organism on an agar culture in a 9cm petri-dish every 2 days until the agar medium was almost 100% covered by mycelia.

2.12.4.2. ISOLATION FROM MYCELIAL SAMPLES

Mycelial samples were divided into small segments and 2-3 pieces were placed in petri-dishes containing 2% A/5% MX. Further culturing and examination was as described in 2.12.4.1.

2.12.4.3. ISOLATION FROM WOOD SAMPLES

"Tear"-shaped segments of wood (~1cm^2) were aseptically removed from the timber and 6 segments per petri-dish were cultured and examined as described in 2.12.4.1.

2.12.4.4. CLEAR "SELLOTAPE" METHOD

A piece of sellotape (0.5cm^2) was placed sticky-side down on a mycelial sample cultured on agar. Using forceps, the sellotape was removed and placed on a microscope slide, fungal side facing upwards and covered with lactophenol-cotton blue dye. A coverslip was placed over the sample which was then viewed for clamp connections using a light microscope.

2.13. ESTIMATION OF PROTEIN CONTENT OF FUNGAL EXTRACTS

5mg of *C. puteana* FPRL 11E lyophilised mycelia was ground to a slurry in 1ml distilled water using a mortar and pestle. The protein content of this sample and other fungal samples were analysed using protein assays described by Lowry *et al.*, 1951; Dewey *et al.*, 1989; Pierce and Swelter, 1977 and Biorad (Biorad-Bulletin 1177EG). The Biorad assay was used for subsequent analysis with the incorporation of

a modified sample hydrolysis step (Walker, personal communication) which was initially described by Lowry *et al.*,(1951).

2.13.1. SODIUM HYDROXIDE SAMPLE HYDROLYSIS

Samples were ground in distilled water as described in 2.13., centrifuged at 13,000 rpm for 10 min at RT and the s/ns decanted for analysis as described in 2.13.2. Pellets were hydrolysed in 1ml 0.05-1.0M sodium hydroxide* at 60°C for 1h, centrifuged at 13,000 rpm for 10 min and the s/ns analysed as detailed below.

2.13.2. BIORAD PROTEIN ASSAY

The determination of protein content by the micro-assay procedure of Biorad was by using Coomassie Brilliant Blue G250 dye reagent (Biorad-Bulletin 1177EG). Protein standards (0-100µl Bovine Serum Albumin (BSA) per ml distilled water) and 160µl dilutions of 0.1-1.0M sodium hydroxide were mixed with 40µl dye reagent in wells of a microtitre plate. Each well was mixed thoroughly by carefully drawing the sample-dye solution into a pipette tip several times. The absorbance was measured at 600nm (since a 595nm filter was not available for the microtitre plate reader) every 10 min for 1h to determine the optimum reaction time for the assay and to estimate any interference of the sodium hydroxide in the assay. All samples were analysed in quadruplicate.

2.14. THE EFFECT OF CULTURE AGE AND OF DIFFERENT SUBSTRATES ON THE MOLECULAR AND IMMUNOLOGICAL NATURE OF *C. PUTEANA*

C. puteana grown on 2% A/5% MX was compared with the same organism grown on 2% A/5% pine sapwood sawdust and on 2% A/5%, 2% A/1%, 2% A/0.2% sodium carboxymethyl cellulose. Cultures of each type were harvested for whole mycelial extracts and exoproteins every 2 days (2.3.1. and 2.3.2.4.) and extracts were prepared for further analysis (2.8.1.).

2.15. WOOD DECAY STUDIES

2.15.1. PREPARATION AND STERILISATION OF WOOD BLOCKS

Sapwood from pine (*Pinus sylvestris*) was cut into blocks of $1cm^3$ dimension and evened off with sandpaper. The blocks were labelled with black ballpoint ink, oven dried at 103°C for at least 3h and the dry weights recorded (Original dry weight). Groups of eight blocks were heat-sealed into polythene bags. All bags were heat-sealed into a second polythene bag and sterilised by gamma irradiation (dose - 2.5 Megarads; Scottish University's Reactor Centre, East Kilbride, Scotland). Sterile blocks were stored in their bags at RT until required.

2.15.2. EXPOSURE OF WOOD BLOCKS TO FUNGAL MYCELIUM

Cultures of *C. puteana* FPRL 11E were grown on 2% A/5% MX in vented screw-topped glass jars until a mycelial mat covered 75% of the agar surface. A thin plastic sterilised support was placed on top of each mycelial mat. Sterile blocks were added to each jar. Direct contact between the blocks and

the agar was avoided by use of the sterile support since this contact can cause excessive moisture uptake by the blocks.

2.15.3. PRODUCTION OF WOOD BLOCKS AT VARIOUS STAGES OF COLONISATION

After decay intervals of 0-12 weeks blocks were harvested and the surface mycelia removed. Blocks were lyophilised o/n as described for fungal mycelia (2.4.), weighed (Final dry weight) and stored until required at -180°C. The percentage weight losses were calculated using the standard formula (Wilkinson, 1979) below;

$$\% \text{ wt loss} = \frac{\text{Original dry wt - Final dry wt}}{\text{Original dry wt}} \times \frac{100}{1}$$

2.15.4. PREPARATION OF WOOD BLOCKS FOR FURTHER ANALYSIS

Frozen wood blocks were ground to a fine sawdust using a Cullato Hammer Mill. For total protein extraction uninfected sawdust samples were prepared in PBS as described for WM proteins (2.3.1.) at a concentration of 50mg per ml of PBS. Infected sawdust samples were prepared in a similar manner at a concentration of 25mg per ml of PBS. For exoprotein preparation, extraction was at the same concentrations as above in 1ml PBS in an eppendorf tube at 4°C o/n with agitation. The suspension was centrifuged at 13,000 rpm for 10 min and the s/n used as a source of exoproteins. Storage of both extracts was at -20°C.

2.15.5. THE EFFECT OF DESICCATION ON THE VIABILITY OF *C. PUTEANA*

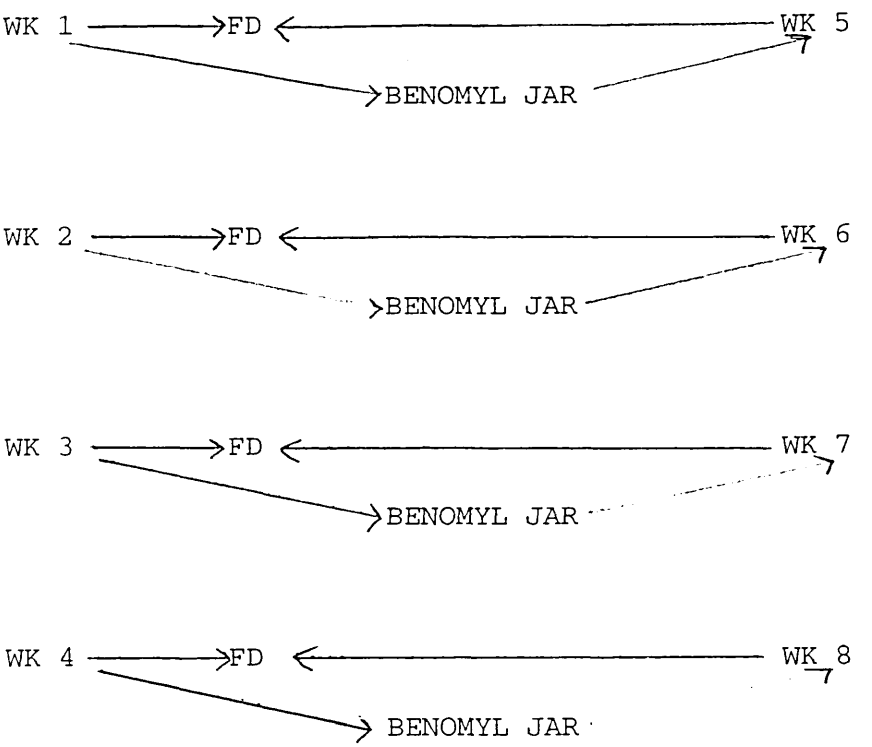
Blocks were harvested at ~30% weight loss (6 weeks, determined in prior analysis). The surface mycelium was removed from one block from each group and the blocks were lyophilised to determine weight losses. The remaining blocks (5 per group) were placed in sterile petri-dishes and desiccated for 1-4 weeks as outlined in Figure 2.1. At intervals of one week, blocks were aseptically removed from the desiccator. 2 blocks were harvested in the normal manner, lyophilised and stored at -180°C. The remaining 3 blocks were re-introduced to a moisture and nutrient rich environment in the form of a culture jar of 2% A/5% MX containing benomyl and streptomycin. After 4 weeks, the blocks were removed from the jar and harvested as described in 2.15.3.

This analysis was repeated to extend the period of desiccation. The method followed was the same as that detailed above, except that 6 control blocks were utilised and each culture jar contained 12 wood blocks. Decayed wood blocks were subjected to 0-12 weeks desiccation followed by the re-introduction of the blocks into a moisture and nutrient rich environment as outlined in Figure 2.2.

2.16. MONOCLONAL ANTIBODY PRODUCTION

The protocol for the preparation of monoclonal antibodies was essentially as described by Galfre and Milstein (1981).

TIME IN DESICCATOR	NUTRIENT ENVIRON.	HARVEST FROM JAR
WK 0*	- FD control blocks and introduce remaining blocks into desiccator	



KEY

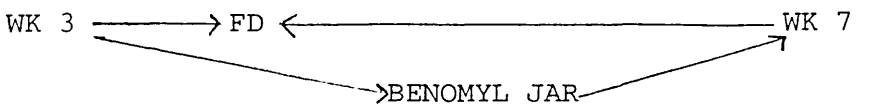
* - Prior to the desiccation analysis all blocks were subjected to decay by *C. puteana* for 8 weeks.

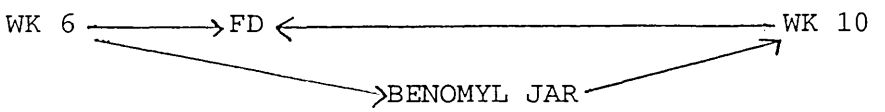
FD - Freeze-dried.

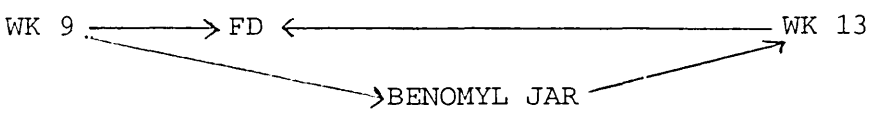
FIGURE 2.1. THE EFFECT OF DESICCATION ON THE VIABLITY OF *C. PUTEANA*; PART I

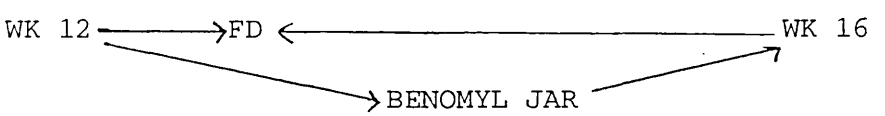
TIME IN DESICCATOR	NUTRIENT ENVIRON. JAR	HARVEST FROM

WK 0* - FD control blocks and introduce remaining blocks into desiccator









KEY

* - Prior to the desiccation analysis all blocks were subjected to decay by *C. puteana* for 8 weeks.

FD - Freeze-dried.

FIGURE 2.2. THE EFFECT OF DESICCATION ON THE VIABILITY OF *C. PUTEANA*; PART II

2.16.1. ANTIGEN PREPARATION

Several antigen preparations were used to produce an immune response in mice. Exoprotein extracts of *C. puteana* FPRL 11E infected sawdust and washings of *C. puteana* FPRL 11E viable mycelia (EP4) were used as immunogens, in addition to the culture filtrate (EP3) and WM extracts of the organism. EP4 antigen preparations were subsequently used in the production of monoclonal antibodies.

2.16.2. IMMUNISATION PROTOCOL

BALB-C mice were immunised with EP4 preparations as outlined in Figure 2.3.

2.16.3. MAINTENANCE OF Sp2/0-Ag14 MOUSE MYELOMA CELLS

Sp2/0-Ag14 mouse myeloma cells (Sp2/0; Flow 05-554) were maintained in 10ml cell growth medium*, RPMI 1640 (1 x concentrate, 100ml including sodium bicarbonate; without L-glutamine) at 37°C. The cells were established by incubation in 5ml growth medium at high cell density and subsequently in 10ml media. Maintenance and expansion of the cell line was achieved by resuspending the cells, transferring 0.5ml to a fresh flask, discarding all but 0.5ml from the original flask and adding 9.5ml growth medium to each flask. Cells were monitored daily by visual inspection using an inverted microscope (Microtec 200, Micro Instruments Ltd.).

DAY PROCEDURE

0 - Primary immunisation Ag, 0.5ml ip

14 - Booster immunisation Ag, 0.5ml ip

18 - Bleed and determine antibody titre of serum

28 - 2nd booster injection if required

32 - Bleed and determine antibody titre of serum

FIGURE 2.3. IMMUNISATION PROTOCOL

2.16.3.1. FREEZING CELLS

Cells were resuspended in 10ml of growth medium, 9.5ml of the suspension centrifuged (1,200 rpm, 10min), and 9.5ml of fresh growth medium fed back to the remaining 0.5ml of cell suspension in the flask. The resultant cell pellet from the centrifugation was resuspended in 0.5ml freezing mixture* and transferred to a cryotube. The cells were frozen at -80°C overnight or on dry ice for 1h., followed by long term storage at -180°C (liquid nitrogen). Supernatants were stored at -20°C where appropriate.

2.16.3.2. THAWING AND RE-ESTABLISHMENT OF CELLS IN CULTURE

A cell aliquot was removed from liquid nitrogen and thawed rapidly at 37°C until most of the frost was melted and immediately placed on ice. The cells were transferred to a 15ml centrifuge tube and 10ml of cold growth medium added slowly to the cells over 5-10 min. The tube was gently inverted and mixed thoroughly. The cells were centrifuged at 1,200 rpm for 5 min and as much media as possible removed. The cells were resuspended in 5ml of growth medium, transferred to a small flask and incubated at high cell density.

2.16.4. BATCH TESTING OF FOETAL CALF SERUM (FCS)

Batch testing was carried out by establishing cloning efficiency in foetal calf serum samples. Specifically, an established Sp2/0 culture was split into a number of flasks (depending on how many serum samples were to be tested, plus one positive control). Cells were passed at least 4 times in the appropriate serum sample prior to cloning.

Passage was at a low density, (normally 0.5ml cells in 9.5ml of medium). After subculture cells were counted and plated out at 5 cells/well and 1 cell/well each density of cells taking up half a microtitre plate. 100% growth was expected at 5 cell/well and >50% growth at 1 cell/well after 7 days incubation at 37°C. To estimate growth each well was scored for growth/no growth. If the FCS was compatible with the Sp2/0 cells, culturing was continued until a sufficient number of cells was obtained for freezing down and storage.

2.16.5. FUSION

2.16.5.1. PREPARATION OF Sp2/0 CELLS

Cells were defrosted and cultured to become established for 3 days. They were then subcultured into an 800ml flask with 30ml growth medium and allowed to grow for a further 3 days. The medium was replaced 24 hours prior to fusion. Immediately prior to fusion, the growth medium was removed, the cells briefly washed with 30ml 0.02% ethylenediamine tetra-acetic acid (EDTA*; to remove excess medium), and then incubated for a few min at 37°C with a fresh 30ml aliquot of EDTA. The cells were knocked off the plastic by gentle pipetting. The cell suspension was centrifuged at 1,500 rpm for 10 min and the EDTA removed. The cells were washed twice in 10ml Dulbecco's A buffer* and counted (2.16.5.3.).

2.16.5.2. PREPARATION OF LYMPHOCYTE CELLS

The selected mouse was boosted 3 days prior to fusion. On the day of the fusion the mouse was killed and the spleen

removed aseptically into 2ml of Dulbecco's A, in a petri dish. Using 21G needles the cells were gently teased from the spleen membrane and transferred in suspension to a 15ml centrifuge tube. The large red cell clumps were allowed to settle, and the cell suspension transferred to a fresh tube and made up to 10ml with Dulbecco's A. The lymphocytes were washed twice in 10ml Dulbecco's A (1,500 rpm, 10 min) and the resultant cell button resuspended in 5ml Dulbecco's A and counted (2.16.5.3.). The expected yield of lymphocyte cells was 1-2 x 10^8 cells.

2.16.5.3. COUNTING CELLS

Viable myeloma and lymphocyte cells, diluted appropriately in Trypan blue dye were counted using a Neubauer Haemocytometer.

2.16.5.4. FUSION PROTOCOL

The cell ratio used was 2-10 lymphocytes cells : 1 myeloma cell. Normally the total number of cells to be used in the fusion was determined by the number of plates to be set up i.e. 2 x 10^5 cells per well, 60 wells per plate, 12ml per plate - 96 well tissue culture plate). Alternatively, the whole spleen was fused, some cells plated out and the remaining cells cultured at 5 x 10^6 per ml in a flask for 10-14 days. The resultant hybrid cells were frozen and stored at -180°C (2.16.3.1.). Prior to proceeding with the fusion protocol, 300µl of each cell type was removed from the centrifuge tubes, for use as controls.

The Sp2/0 and lymphocyte cells were mixed together in a 50ml centrifuge tube, the volume made up to 30ml with

Dulbecco's A and spun at 1,200 rpm for 8 min. All of the supernatant was carefully decanted and the pellet gently tapped loose. 1ml polyethylene glycol (PEG*; pH 9.0) pre-warmed to 37°C in an incubator was added and the tube swirled to resuspend the cells. The cells were spun at 800 rpm for 5 min. 8 min after the addition of PEG, 5ml of Dulbecco's A was gently added by layering over the cell pellet. The cells were resuspended by gentle swirling, spun at 1,200 rpm for 5 min and the supernatant decanted. 5ml Hypoxanthine-aminopterin-thymidine (HAT; Flow)/growth medium* was added slowly without disturbing the cell pellet (the HAT/growth medium was prepared 2 days prior to use to check for contamination). The tube was left for 5-7 min and swirled to resuspend the cells (gentle pipetting was sometimes necessary to ensure resuspension of the pellet).

The cells were diluted to 1 x 10^{-6} per ml in HAT/growth medium, and plated out at 200µl per well (flat-bottomed 96-well plate, inner wells only). Controls of myeloma and lymphocyte cells were included at concentrations equivalent to that used in the fusion (only a few wells were required). The controls were fed with HAT/growth medium at the same time as the hybrids. The lymphocyte cell control was used to check for background antibody production in the screening assay. Controls were regularly checked to ensure that myeloma cells were sensitive to the HAT/growth medium.

2.16.6. FEEDING HYBRIDS

3-4 days after fusion one-two drops of HAT/growth medium was added to each well. Thereafter, wells were fed by removing half the contents and adding back 100µl HAT/growth medium every 3-4 days. Wells were scanned after 7-10 days

for the first signs of hybrid colonies and the approximate number of colonies per well noted. Once hybrids were covering about half the well area in total and/or the medium appeared yellow, the supernatant was tested for specific antibody content. The cells were not fed during the preceding three days prior to testing to allow the antibody concentration to increase. After testing selected hybrids were cloned immediately.

2.16.7. CLONING HYBRIDS

Hybrid cells were resuspended with a fine-tipped pipette and 10µl removed for Trypan blue viability testing and counting. Remaining cells were transferred to 3ml Hypoxanthine-Thymidine (HT; Flow)/growth medium* in a small flask, returning a few drops of diluted cells to the well as a back up.

A volume of growth medium containing 150 cells was added to a centrifuge tube containing 6ml of medium to give 25 cells/ml. The cells were mixed thoroughly and 1ml transferred to a tube containing 4ml of growth medium (5 cells/ml). 200µl was dispensed from the 25 cells/ml tube into the top two rows of a round bottomed plate and 200µl of the 5 cells/ml suspension dispensed into the second two rows of the plate.

After 5-7 days, the wells were inspected for cell growth and the number of colonies growing in each well recorded. From these recordings, wells containing single colonies were determined and tested for specific antibody production. Multiples were tested if all singles were negative or if no single hybrids were apparent. If cell

growth was slow, fresh cloning medium was provided after 7-10 days, but cells were not fed for three days prior to testing.

If only one or two of the clones tested were positive, this indicated either that the cell line was unstable or that the original well contained a mixture of positive and negative cells. In either case, recloning using the best clone was necessary, plating out a 96-well plate at 1 cell/well. If less than 90% of clones were positive then the procedure was repeated until >90% were positive.

2.16.8. EXPANSION OF CLONES

Appropriate clones were transferred to small flasks containing 3ml HT/growth medium (+ another 10% FCS, if the clone was slow growing). The wells of the microtitre plate were fed back with 100µl of HT/growth medium as a back up. After 2 days, a further 2ml HT/growth medium were added to the flasks and when the cells were growing at high density they were frozen in liquid nitrogen. The flask was fed back with growth medium and cells were repeatedly frozen down until 6-8 vials were stored. Supernatants from freezings were saved and checked for antibody production.

2.17. SCREENING FOR ANTIBODY PRODUCTION;CROSS-REACTIVITY TESTING, USING EIA

All antigen extracts were prepared as described in section 2.3. The EIA methodology was as described in section 2.7. except that Ab2 was HRP-labelled anti-mouse gamma globulin (diluted 1:500; v/v, in PBS/5%NCS/0.05%tw) and primary antibodies were undiluted hybrid s/n. Controls used for

antibody screening were growth medium and control lymphocyte and myeloma cell s/n; for cross-reactivity studies controls of growth medium and pre-immune sera were utilised. For cross-reactivity, columns (1-11) of the microtitre plates contained individual fungal antigen extracts leaving column 12 free for use as a no antigen control to test for non-specific binding of Ab1 or Ab2.

2.18. ISOTYPING MONOCLONAL ANTIBODIES

The monoclonal antibodies produced were isotyped using the Mouse Monoclonal Isotyping Kit supplied by Amersham. The method was according to the detailed protocol available in the instruction booklet provided (RPN 29). The typing stick was incubated with 3ml undiluted hybrid s/n for 15 min at RT with agitation. The stick was washed twice (5 min per wash) in 5ml tris buffered saline (TBS-T*). Incubation with 3ml HRP-Ab2 (diluted 1:500 in TBS-T; v/v) was for 15 min at RT with agitation followed by 5 min washes as described above. 3ml of substrate solution* was added and the stick incubated for 15 min. The substrate solution was discarded and the stick washed in 3 changes of 5ml distilled water (2-3 min per wash). Results were interpreted prior to air-drying.

2.19. DETECTION OF *C. PUTEANA*

2.19.1. ORIGIN OF FIELD ISOLATES

The frigate *Unicorn* and RRS Discovery are maritime artifacts which are currently under the protection of the *Unicorn* Preservation Society and Dundee Industrial Heritage, respectively. The historic ships are subject to

fungal decay caused by wet rot organisms and since both add to the tourist industry of the City, Dundee District Council provided financial support for the research described in this thesis. Consequently, the *Unicorn* Preservation Society and Dundee Industrial Heritage allowed access to the ships thus providing a supply of field samples for this project.

2.19.2. ISOLATION OF MYCELIAL/STRAND SAMPLES

Mycelial and strand samples found growing on the timber of the ships were removed to labelled resealable plastic bags, using a scalpel flamed in 70% ethanol. The samples were described and small pieces were cultured and harvested as described in 2.12.4. Pure cultures were examined for clamp connections and thus the presence of basidiomycetes (2.12.4.4.). The location of these samples on the frigate *Unicorn* are detailed in Figure 2.4.

2.19.3. SAMPLING OF WOOD CORES

5mm cores of wood were aseptically extracted from the timbers of the *Unicorn*. Details of the number and location of cores removed are shown in Figure 2.5. Selected cores were divided into 2 segments; one segment was split into small pieces and microbiological isolation of the infecting organism was carried out (section 2.12.4.3.). The resultant mycelium or whole core, was harvested and lyophilised (2.3.1. and 2.4.). The second segment was ground to a fine powder using a Culatto Hammer Mill and exoprotein extracts were prepared for analysis (2.15.4.).

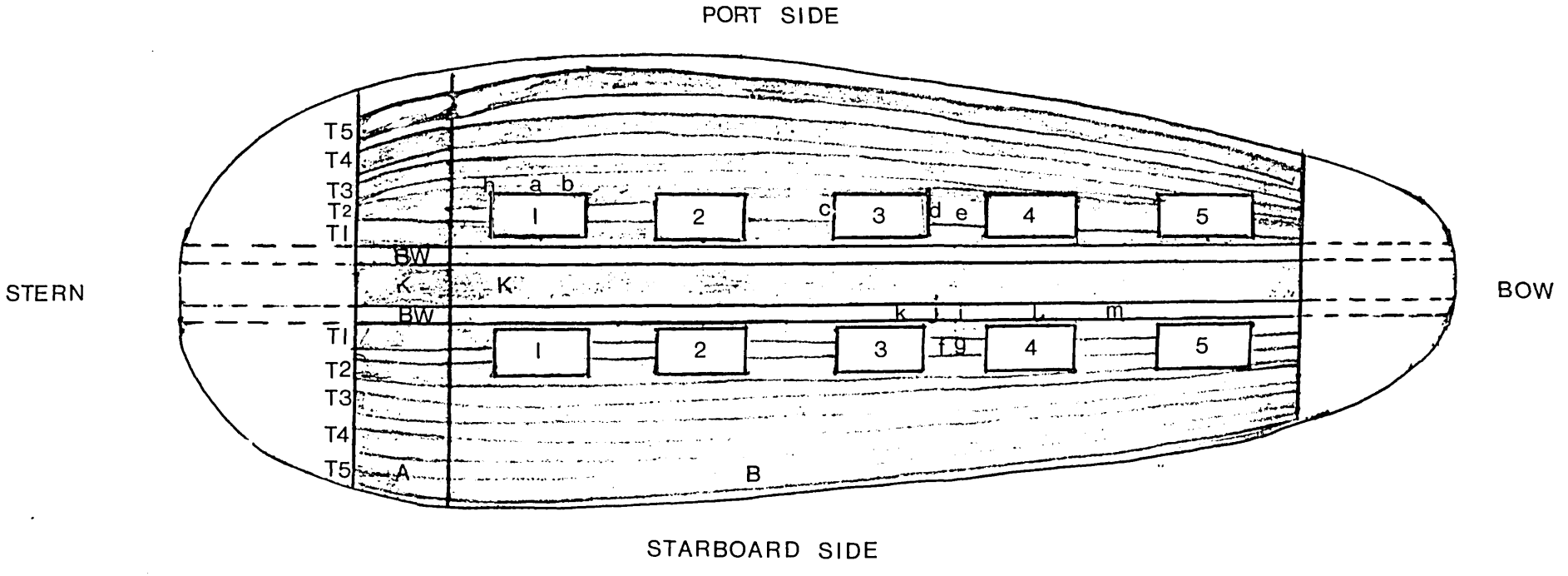


FIGURE 2.4. DIAGRAMMATIC SKETCH OF THE *UNICORN* INDICATING THE LOCATION OF MYCELIAL/STRAND FIELD SAMPLES

KEY

T1-T5 - Thick-stuff* 1-5 up to water line
1-5 - Stacks of iron making up ballast
a-m - Strand and mycelial samples

A - Aft hold
B - Forward hold
K - Keelson+
BW - Bilge water

* - Thick-stuff; large structural timbers spanning the ship from bow to stern
\+ - Keelson; main spine of ship, located directly above keel.

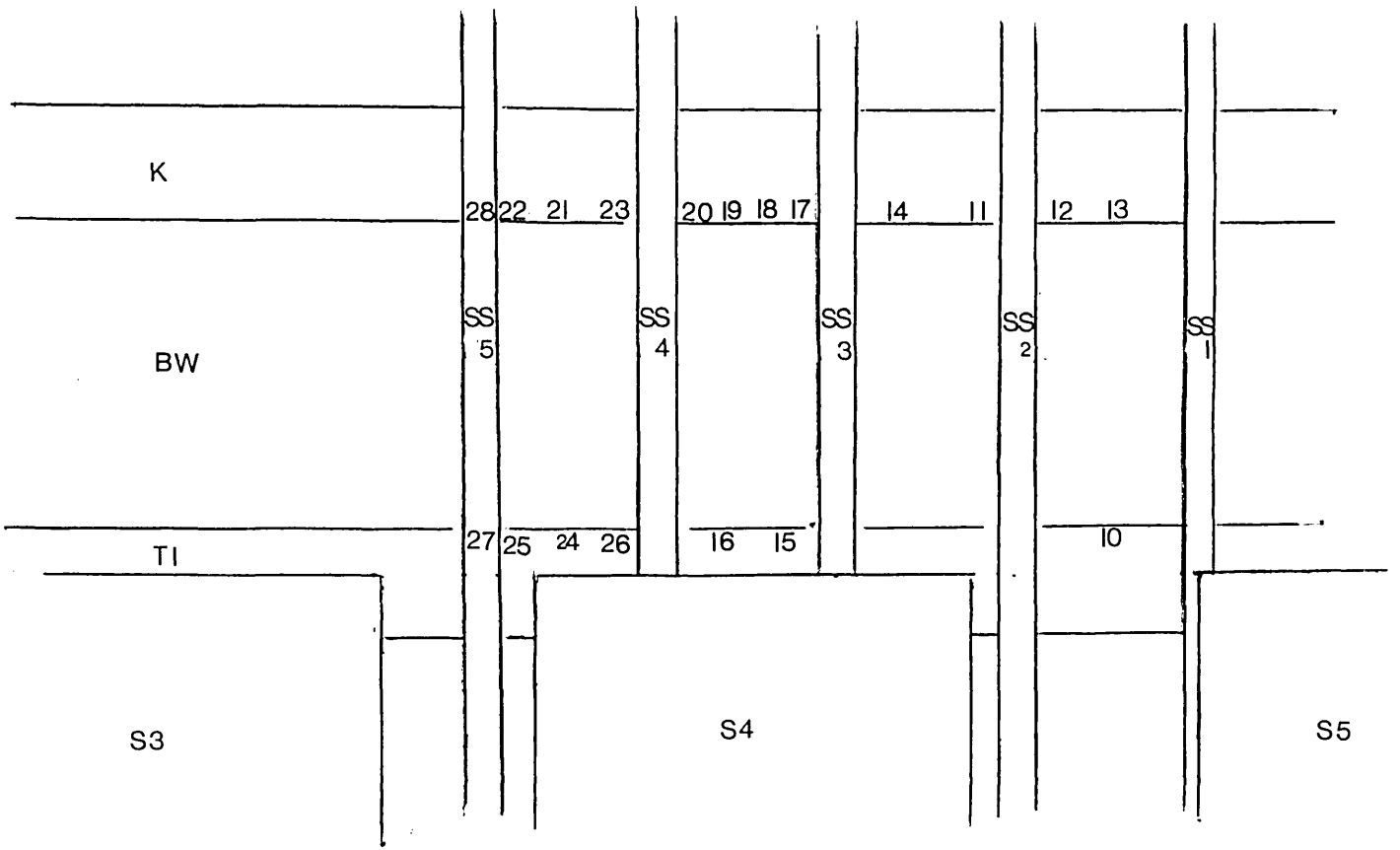


FIGURE 2.5. DIAGRAMMATIC SKETCH OF THE FORWARD HOLD OF THE *UNICORN* INDICATING THE LOCATION OF WOOD CORE SAMPLES

KEY

S3-5 - Stacks 3-5
SS1-5 - Stack supports 1-5
10-28 - Cores 10-28
K - Keelson
BW - Bilge water
T1 - Thick-stuff 1

NB. Cores 1-9 were all removed from Thick-stuff 2 at stack 1 on the port side of the ship (see Figure 2.4.)

CHAPTER THREE

IDENTIFICATION OF *C. PUTEANA*

3.1. INTRODUCTION

This chapter is concerned with the development and analysis of molecular and immunological identification methods for *C. puteana* as possible alternatives to conventional methods for the identification of wood decay fungi. The techniques studied involved SDS-PAGE analysis of fungal proteins and utilisation of EIA and western blotting. Salient features of the theory of these techniques are discussed in the following sections. The results are presented as studies of two different extracts of *C. puteana* and a range of different wood inhabiting fungi. The extracts of mycelia are soluble extracts of whole freeze-dried mycelia (WM; 3.2., 3.3. and 3.4.) and buffer soluble surface washings of viable mycelia (SW; 3.5., 3.6. and 3.7.). The preparation of these extracts are as described in 2.3.1. and 2.3.2.4.

3.1.1. SDS-PAGE

SDS-PAGE is the electrophoretic separation of soluble proteins on the basis of their molecular weight. After separation proteins are immobilised in a polyacrylamide gel matrix and visualised using a suitable method, e.g. the highly sensitive silver staining method of Switzer *et al.*, (1979), recently improved by Blum *et al.*, 1987. The determination of the molecular weights of the proteins can be achieved by the separation of proteins of known molecular weights in test gels.

3.1.2. EIA

Several types of enzyme immunoassay have been developed in order to fulfill different assay requirements (reviewed in Engvall, 1980; Voller and Bidwell, 1980; Edwards, 1985). During this project a multi-layered enzyme immunoassay was employed and the salient features of the technique are presented in Figure 3.1., which shows a solid phase antigen EIA. The immunosorbent, an immobilised antigen relevant to the particular antibody to be assayed, is reacted with antibody contained in a primary antiserum. Subsequent reaction of the primary antiserum with enzyme-labelled anti-immunoglobulin antibodies, in conjuction with an appropriate chromagen system, allows the detection of low concentrations of antibodies. Depending upon the assay requirements, detection of antigen using specific concentrations of antibodies is also possible.

3.1.3. WESTERN BLOTTING

Western blotting involves the separation of proteins by SDS-PAGE followed by their electrophoretic transfer onto a protein binding membrane. Immobilised proteins are then stained by incubation with a primary antiserum followed by subsequent detection of the bound antibodies by an enzyme-labelled immunoglobulin:chromagen system.

3.2. WM EXTRACT: ANALYSIS OF *C. PUTEANA* AND OTHER FUNGI KNOWN TO INHABIT WOOD, BY SDS-PAGE

The technique of SDS-PAGE was carried out as described in 2.8. The reference fungus used throughout this project was

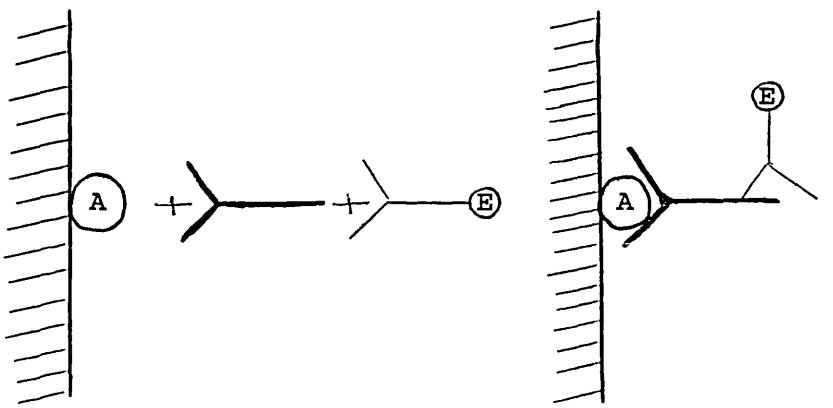


FIGURE 3.1. Diagrammatic representation of the solid phase antigen EIA, for detecting antibodies. Key: **A** = antigen; Y = antibody; Y = anti-immunoglobulin antibody and **E** = enzyme label. The bound enzyme-linked antibodies were detected with a suitable chromagen system.

C. puteana strain FPRL 11E. Table 3.1. shows a list of fungal organisms which were used in the study described in this chapter. Also in Table 3.1. are abbreviations used in subsequent tables in this chapter. All fungal preparations used in the study reported in this section were soluble extracts of whole mycelia (WM) unless otherwise stated.

3.2.1. ANALYSIS OF MEMBERS OF THE GENUS *CONIOPHORA*

3.2.1.1. VISUAL INTERPRETATION OF PROTEIN PROFILES

A reproducible protein profile for the reference organism *C. puteana* FPRL 11E was established as the basis for subsequent molecular analysis (Figure 3.2., even numbered tracks). To determine if this profile was unique to FPRL 11E or if it was similar to those for other strains of *C. puteana* and other species of *Coniophora*, protein profiles of such organisms were prepared and are shown in Figure 3.2. The data indicate that the profiles of members of the genus *Coniophora* are similar to that of FPRL 11E. However, differences between the profiles do exist specifically in the area of the gel representing proteins of molecular weight between 45,000 and 24,000 Da. The minor differences which are apparent allow differentiation of the strains of *C. puteana*.

Analogous to this, the species of *Coniophora* can also be distinguished by the presence or absence of particular proteins. For instance, *C. arida* FPRL 411 (track 15) has the unique protein of approximate molecular weight of 42,600 Da; *C. marmorata* FPRL 410 (track 13) has a unique protein of approximate molecular weight of 33,800 Da and C.

BROWN ROT FUNGI

11E - *Coniophora puteana* FPRL 11E
11A - *Coniophora puteana* FPRL 11A
11B - *Coniophora puteana* FPRL 11B
11Q - *Coniophora puteana* FPRL 11Q
B15 - *Coniophora puteana* BAM 15
CA - *Coniophora arida* FPRL 411
CM - *Coniophora marmorata* FPRL 410
U20 - Isolate DIT-U20
SL - *Serpula lacrymans* FPRL 12C
PP - *Poria placenta* FPRL 280
FV - *Fibroporia vaillantii* FPRL 14H
AX - *Amyloporia xantha* FPRL 62F
LS - *Laetiporus sulphureus* FPRL 29
GT - *Gloeophyllum trabeum* BAM 109
GS - *Gloeophyllum sepiarium* FPRL 10D
LL - *Neolentinus (Lentinus) lepideus* FPRL 7F
DQ - *Daedalea quercina* FPRL 38
PG - *Peniophora gigantea* FPRL 175B
SP - *Leucogyrophana (Serpula) pinastri* FPRL 141B
Pp - *Paxillus panuoides* FPRL 8B
PI - *Poria incrassata* FPRL 71

WHITE ROT FUNGI

PO - *Pleurotus ostreatus* FPRL 40A
SC - *Schizophyllum commune* FPRL 9
DC - *Daldinia concentrica* FPRL 26E
SS - *Stereum sanguinolentum* FPRL 27D
CV - *Coriolus versicolor* MAD 697
Cp - *Ceratocystis picea*
HA - *Heterobasidion annosum* FPRL 41E
V - *Verticillium* spp. NRS 69.0

NON-BASIDIOMYCETES

TH - *Trichoderma harzianium* IMI 206040
PV - *Paeciliomyces variotii* DIT pi
CR - *Cladosporium resinae* BM 13385-1-22A
TP - *Trichoderma polysporium* IMI 206039

FURTHER ABBREVIATIONS

WM - Whole Mycelial preparation
SW - Surface mycelial washings
REF - Organism to which others are compared
TEST - Organism which is compared to REF

TABLE 3.1.
FUNGAL ORGANISMS AND ABBREVIATIONS USED IN CHAPTER 3

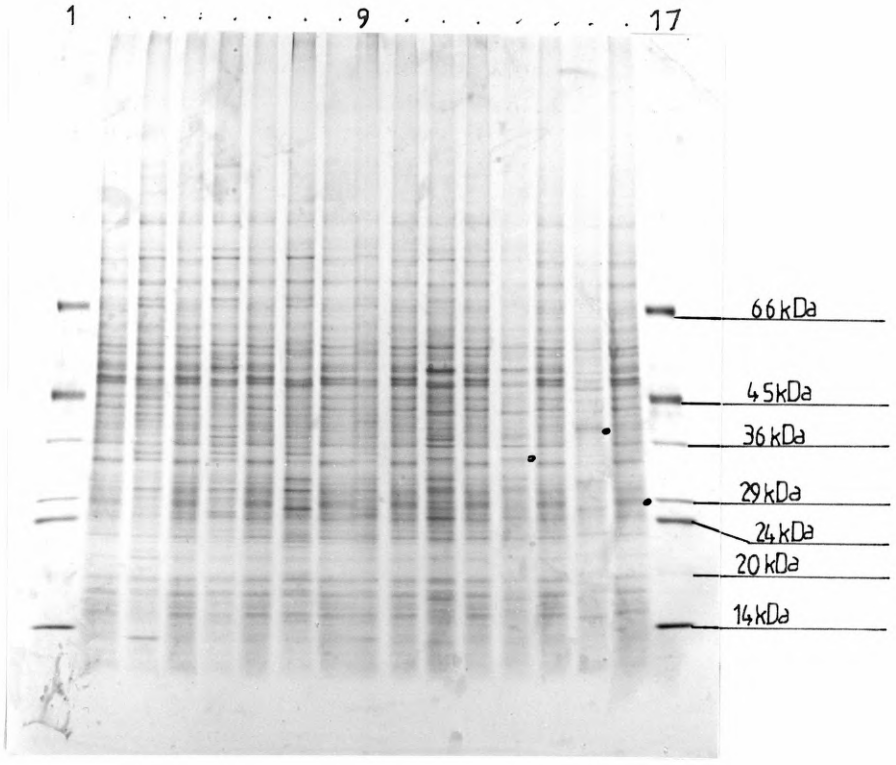


FIGURE 3.2. SDS-PAGE analysis of members of the *Coniophora* genus. Tracks represent: 1, 17 - Standard molecular weight markers (MWM); 2, 4, 6, 8, 10, 12, 14, 16 - *C. puteana* FPRL 11E; 3 - *C. puteana* FPRL 11A; 5 - *C. puteana* FPRL 11B; 7 - *C. puteana* FPRL 11Q; 9 - *C. puteana* BAM 15; 11 - isolate DIT-U20; 13 - *C. marmorata* FPRL 410; 15 - *C. arida* FPRL 411. In this and all subsequent figures the molecular weight markers used were of the following sizes, 14,200, 20,100, 24,000, 29,000, 36,000 45,000 and 66,000 Daltons.

puteana (even numbered tracks) has a unique protein of approximate molecular weight of 29,000 Da.

3.2.1.2. PERCENTAGE SIMILARITY INDEX ANALYSIS FOR THE INTERPRETATION OF SDS-PAGE GELS

Visual interpretation of the data produced on gels indicated that the strains of *C. puteana* FPRL 11E are more similar to each other than to other species of *Coniophora*. This information can be confirmed by numerical analysis for example by the calculation of Percentage Similarity Indices. Table 3.2. indicates the percentage of protein bands in common with *C. puteana* FPRL 11E for the organisms shown in Figure 3.2. The results show that the organism most similar to FPRL 11E is *C. puteana* FPRL 11A. Organisms FPRL 11B, 11Q and BAM 15 are also highly similar to FPRL 11E, with *C. arida* and *C. marmorata* being the most different. As well as organisms within the species of *Coniophora* obtained from established culture collections, Figure 3.2. also shows the protein profile for a recent isolate from an apparent outbreak of *C. puteana* (isolate DIT-U20, track 11; details in chapter 6). Similarity index measurements for this isolate produced a mean value of 60% (Table 3.2.) when compared to *C. puteana* strains indicating its probable identity as a member of the genus *Coniophora*. However on comparison of the isolate with *C. arida* and *C. marmorata*, similarities of 68% and 91% respectively were obtained suggesting that DIT-U20 is in fact an isolate of *C. marmorata*.

(i) MEMBERS OF THE GENUS *CONIOPHORA*

TEST REF	11A	11B	11Q	B15	CM	CA	U20	CP	MEAN
11E	83	70	73	75	52	54	60		75

(ii) IDENTITY OF ISOLATE DIT-U20

TEST REF	11E	11A	11B	11Q	B15	CM	CA	CP	MEAN
U20	60	62	61	54	63	91	68		60

TABLE 3.2.

PERCENTAGE SIMILARITY INDICES FOR MEMBERS OF THE GENUS *CONIOPHORA* COMPARED TO FPRL 11E (i) AND TO ISOLATE DIT-U20 (ii)

To check if bias was introduced by the use of one organism as a reference, similarity indices were constructed using each organism as a reference (Table 3.3.). As each strain of *C. puteana* was analysed a distinction between *C. puteana* and other species was revealed. Intra-species similarity indices for *C. puteana* were 72-80% and inter-species similarity indices for *Coniophora* species were <63%. At this stage the strains of *C. puteana* did not fall into distinct groups, however such groups did become apparent on further analysis (3.4.1.).

Using *C. marmorata* and *C. arida* as references produced similar results in that these two organisms could be differentiated from all strains of *C. puteana* tested. The similarity between U20 and *C. marmorata* was confirmed in this analysis.

TEST / REF	11E	11A	11B	11Q	B15	U20	CM	CA	CP MEAN
11E	100	83	70	73	75	60	52	54	76
11A	74	100	79	71	74	62	49	52	75
11B	75	81	100	81	73	61	47	51	78
11Q	67	73	66	100	81	54	48	50	72
B15	76	82	76	84	100	63	49	54	80
U20	75	75	69	67	75	100	67	54	
CM	62	76	67	76	67	91	100	52	
CA	77	73	77	73	77	68	64	100	

TABLE 3.3.

PERCENTAGE SIMILARITY INDEX FOR MEMBERS OF THE GENUS *CONIOPHORA* ANALYSED BY SDS-PAGE

3.2.2. ANALYSIS OF A RANGE OF FUNGI KNOWN TO INHABIT WOOD

A comparison of SDS-profiles of FPRL 11E with a range of basidiomycetes known to inhabit wood is shown in Figure 3.3. Although a number of organisms share common protein bands, the overall pattern of proteins is apparently unique to each organism. A set of percentage similarity indices for the profiles shown in Figure 3.3. is displayed in Table 3.4. and confirms the visual analysis of the results. Indices for the comparison of all fungi with FPRL 11E were not greater than 46%, with an index value for isolate DIT-U20 of 64%. Similarly when other organisms were used as references and compared to the remaining fungi, it was possible to distinguish between all the organisms on the basis of similarity indices.

3.3. WM EXTRACT: INVESTIGATION OF ANTISERUM 88/1

Section 3.2. covered the SDS-PAGE analysis of fungi known to inhabit wood. The possibility of using immunological analysis to discriminate between these fungi was investigated with antiserum 88/1. Antigen and antibody dilution experiments are described in the next 2 sections; optimal concentrations of antigen and antibody were selected from the results of these experiments.

3.3.1. ANTIGEN DILUTION CURVE

Two-fold serial dilutions of a PBS-soluble antigen preparation of WM, the initial concentration of which was 100µg per ml of PBS, were tested using EIA. The primary unlabelled antiserum was diluted 1:100 (v/v) in PBS/0.05% Tween 20. An antigen dilution curve was constructed for the

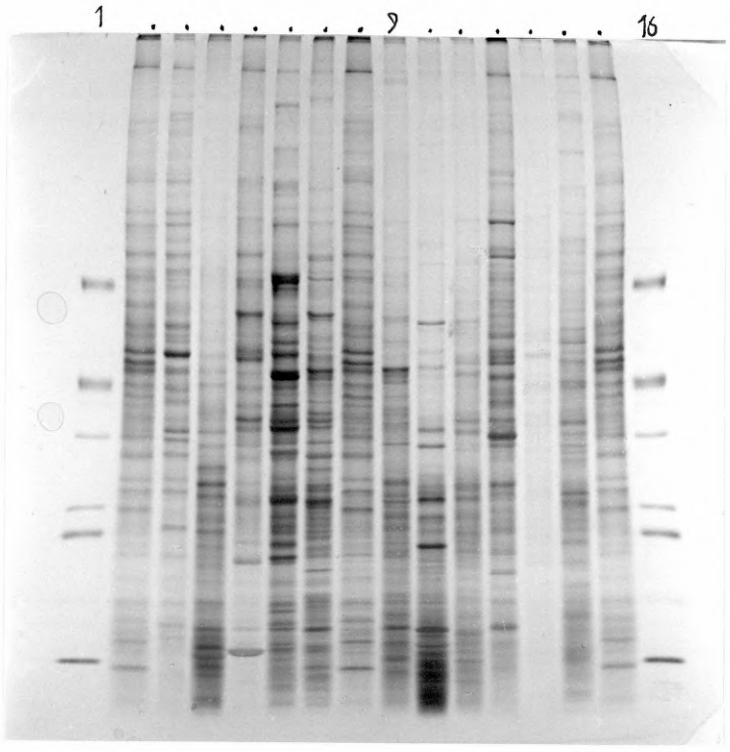


FIGURE 3.3. SDS-PAGE analysis of various wood inhabiting fungi. Tracks represent: 1, 16 - MWM (Figure 3.2.); 2, 8, 15 - *C. puteana* FPRL 11E; 3 - isolate DIT-U20; 4 - *S. lacrymans* FPRL 12C; 5 - *P. placenta* FPRL 280; 6 - *F. vaillantii* FPRL 14H; 7 - *A. xantha* FPRL 62F; 9 - *L. sulphureus* FPRL 29; 10 - *G. trabeum* BAM 109; 11 - *G. sepiarium* FPRL 10D; 12 - *H. annosum* FPRL 41E; 13 - *Verticillium* spp.; 14 - *T. harzianium* IMI 206040.

REF \ TEST	CP	U20	SL	PP	FV	AX	LS	GT	GS	HA	V	TH
CP	100	64	36	24	46	40	26	24	24	29	7	38
U20	62	100	22	22	30	38	24	11	11	35	14	43
SL	41	27	100	22	56	43	38	41	24	30	16	30
PP	30	33	22	100	56	48	30	19	33	44	19	37
FV	40	26	48	32	100	40	42	26	20	32	14	30
AX	33	33	39	26	48	100	33	33	24	41	7	28
LS	41	29	38	32	41	38	100	26	24	27	15	32
GT	29	14	46	21	36	39	36	100	43	25	4	25
GS	36	16	24	20	40	32	36	44	100	24	20	20
HA	33	33	25	33	33	45	25	20	20	100	8	23
V	18	36	36	27	45	27	36	18	45	27	100	18
TH	42	38	36	25	36	36	33	22	17	27	5	100

TABLE 3.4.

PERCENTAGE SIMILARITIES FOR A RANGE OF FUNGI KNOWN TO INHABIT WOOD ANALYSED BY SDS-PAGE

antiserum to determine the optimum antigen dilution. Figure 3.4. shows the antigen dilution curve obtained for antiserum 88/1 and for pre-immune serum (normal rabbit serum; NRS). Subsequently, a 1:2 (v/v) dilution of the WM antigen preparation was used in further applications of the assay, since this dilution gave the greatest difference between the primary antiserum and the pre-immune control. This dilution represents a concentration of 50µg per ml of extract, or 5µg per well of the EIA plate.

3.3.2. ANTIBODY DILUTION CURVE

An antibody dilution curve was constructed by testing two-fold serial dilutions of antiserum 88/1. The titre of the antiserum, the highest antibody dilution showing 100% antigen binding was assessed from the antibody dilution curve (Figure 3.5.). The titre for antiserum 88/1, 1:200 (v/v) was subsequently used in further investigations.

3.3.3. SPECIFICITY OF ANTISERUM 88/1

The specificity of antiserum 88/1 was tested against a variety of WM antigens from fungi known to inhabit wood (Figure 3.6.). The results indicate that the antiserum cross-reacted substantially with only 8 of the 22 fungi tested, 5 of the remaining 14 showing limited cross-reactivity. All 8 of the major cross-reacting fungi are basidiomycetes and only one of these occupies the same ecological niche as *C. puteana* viz., *A. xantha*.

Assays to this point were analysed at 405nm, however all subsequent assays were analysed at 450nm (Figure 3.7.).

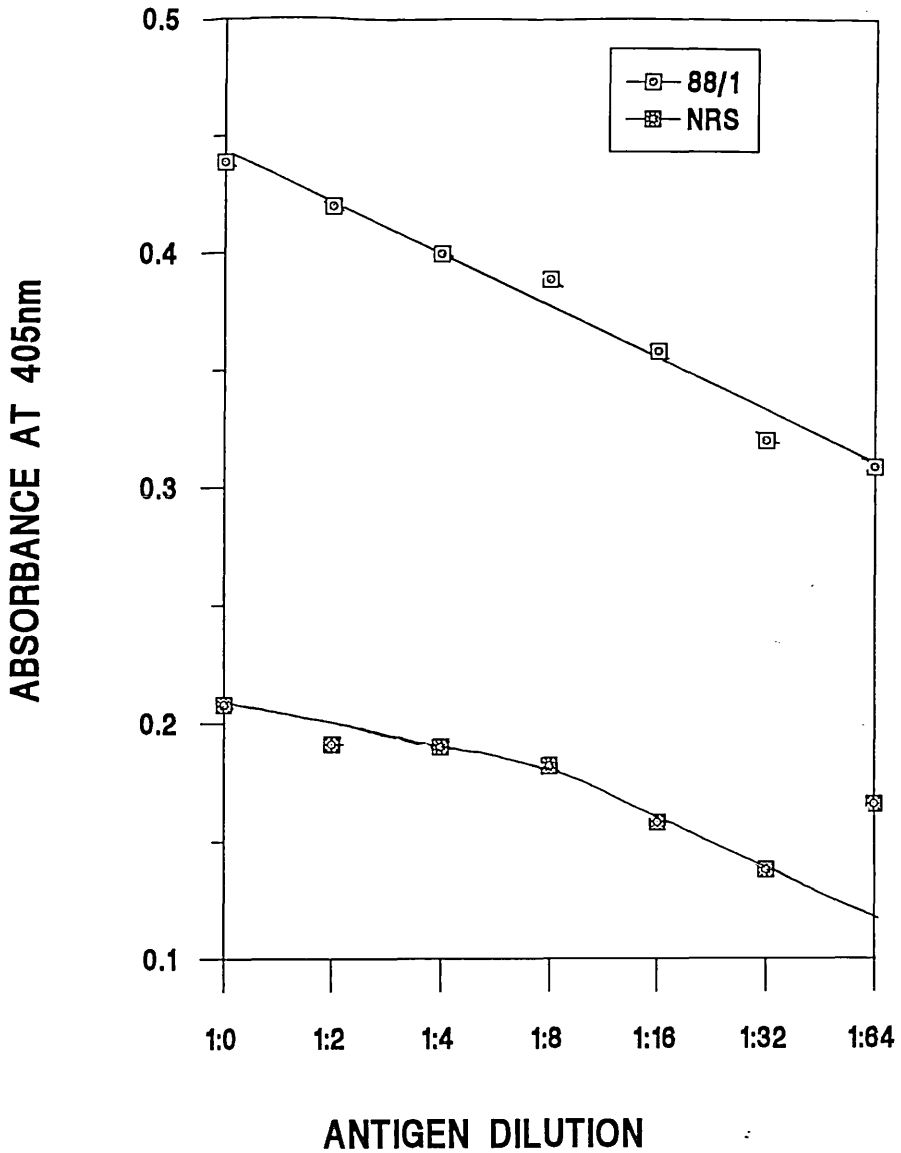


FIGURE 3.4. ANTIGEN DILUTION CURVE (INITIAL CONCENTRATION OF ANTIGEN 100ug per ml PBS)

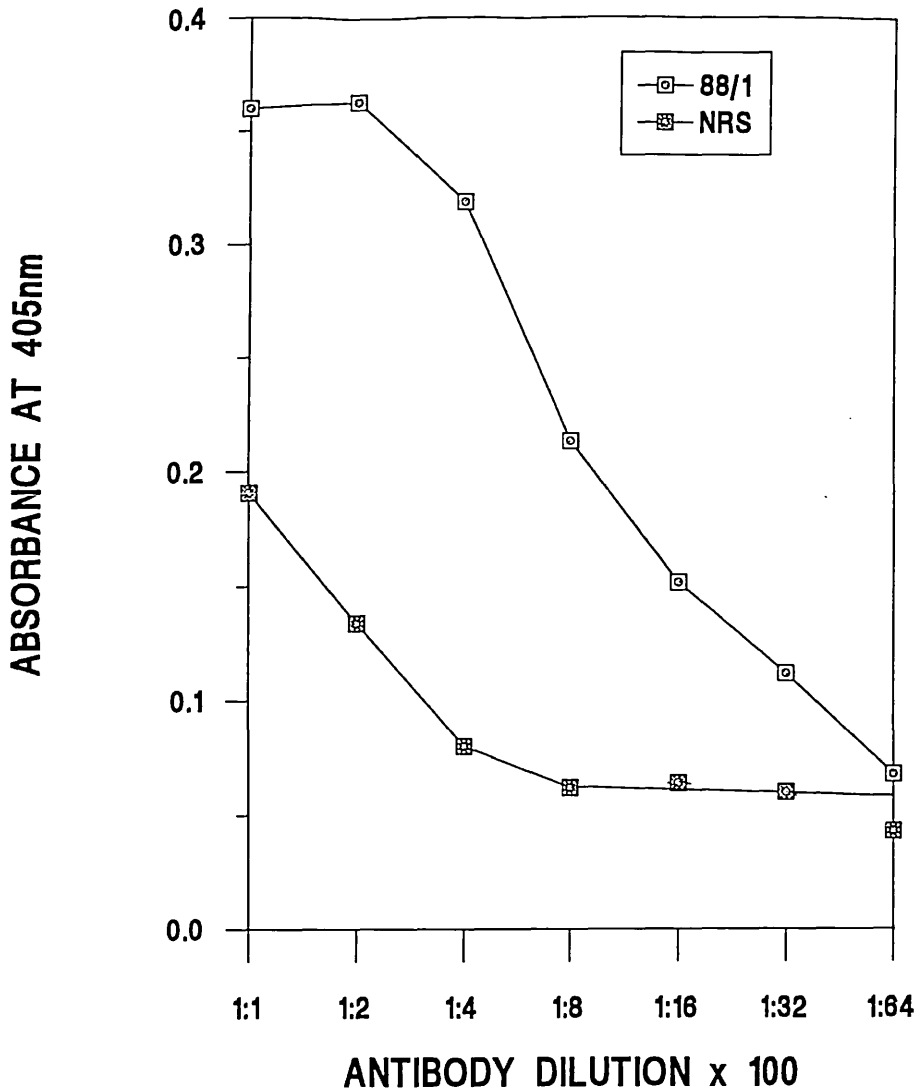


FIGURE 3.5. ANTIBODY DILUTION CURVE FOR ANTISERUM 88/1

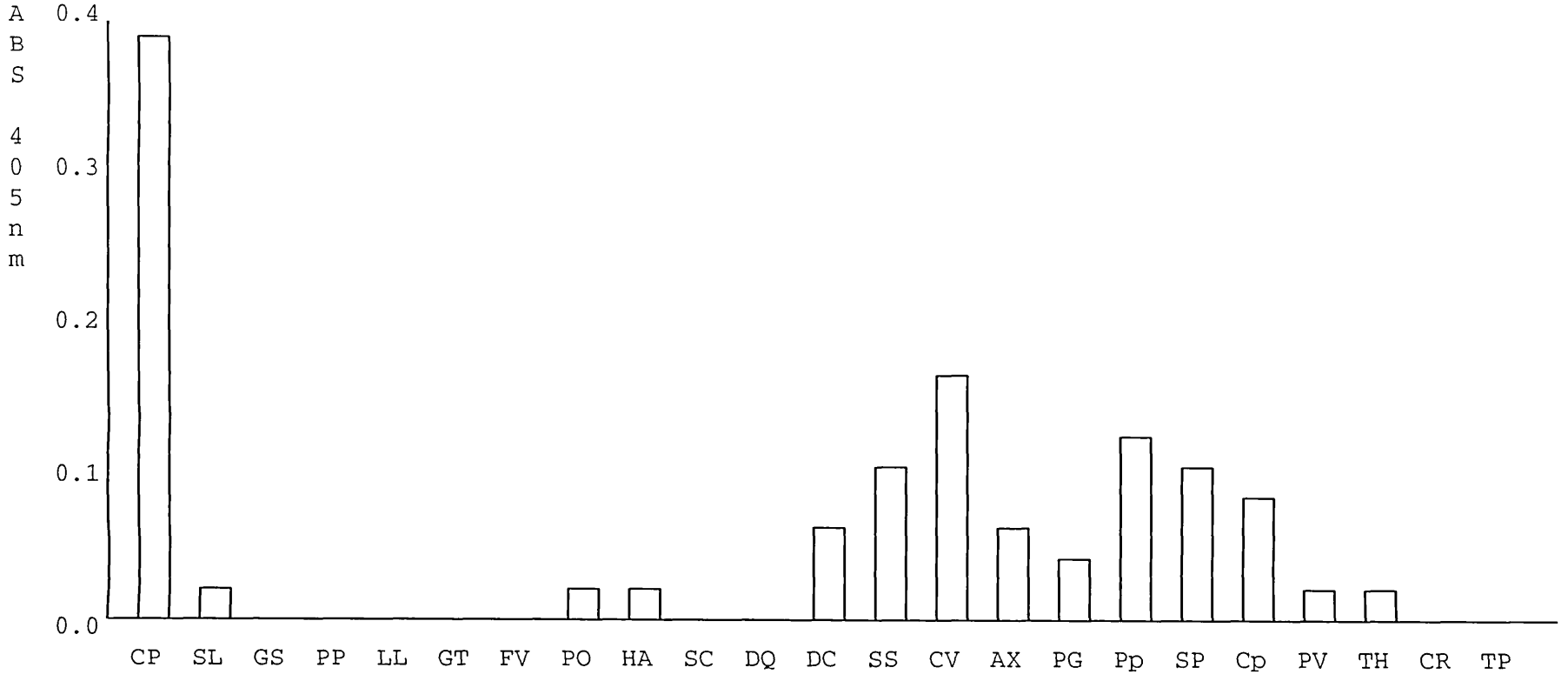


FIGURE 3.6 - CROSS-REACTIVITY OF ANTISERUM 88/1 WITH A RANGE OF FUNGI KNOWN TO INHABIT WOOD

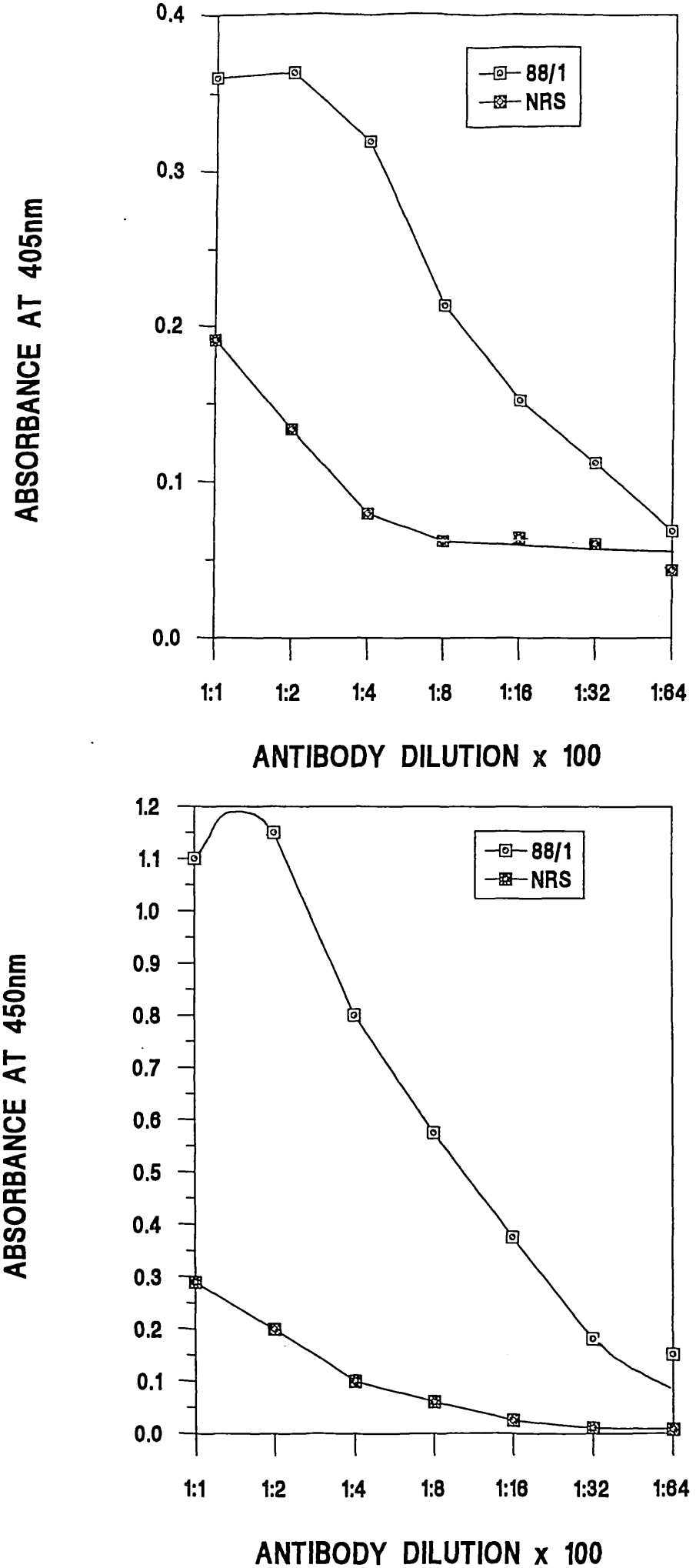


FIGURE 3.7. COMPARISON OF ANTIBODY DILUTIONS AT DIFFERENT WAVELENGHTHS

3.4. WM EXTRACT: ANALYSIS OF *C. PUTEANA* AND OTHER FUNGI KNOWN TO INHABIT WOOD, BY WESTERN BLOTTING

The technique of western blotting was as described in 2.10. and antiserum 88/1 was used.

3.4.1. ANALYSIS OF MEMBERS OF THE GENUS *CONIOPHORA*

The antigenic profiles for members of the *Coniophora* genus are shown in Figure 3.8. The reference organism, *C. puteana* FPRL 11E possesses 5 major antigens which are evident after blotting with antiserum 88/1. They have approximate molecular weights of: antigen no. 1 (Ag 1) - 136,500 Da; Ag 2 - 98,900-92,300 Da; Ag 3 - 68,400-65,300 Da; Ag 4 - 39,500-37,000 Da and Ag 5 - 32,000 Da. For each strain of *C. puteana* and for each *Coniophora* species there is an antigen present in the same molecular weight range as Ags 1-5 of *C. puteana* FPRL 11E. A set of common antigens (general profile) for the members of the *Coniophora* genus tested therefore exists, although the antigens may not share exactly the same molecular weights.

Although there is an overall antigenic profile for the genus *Coniophora*, four specific profiles are apparent. Profile A is shown by FPRL 11E (even numbered tracks) and FPRL 11A (track 15), profile B by BAM 15, FPRL 11Q and FPRL 11B (tracks 9, 11, 13), profile C by *C. marmorata* and isolate U20 (tracks 5, 7) and profile D by *C. arida* (track 3). These profiles were numerically analysed (Table 3.5.; 2.12.2.) and the results confirm the presence of 4 profiles within the *Coniophora* genus. In addition, each organism tested shares at least 2 antigens with FPRL 11E. The similarity between *C. marmorata* and U20, confirms the

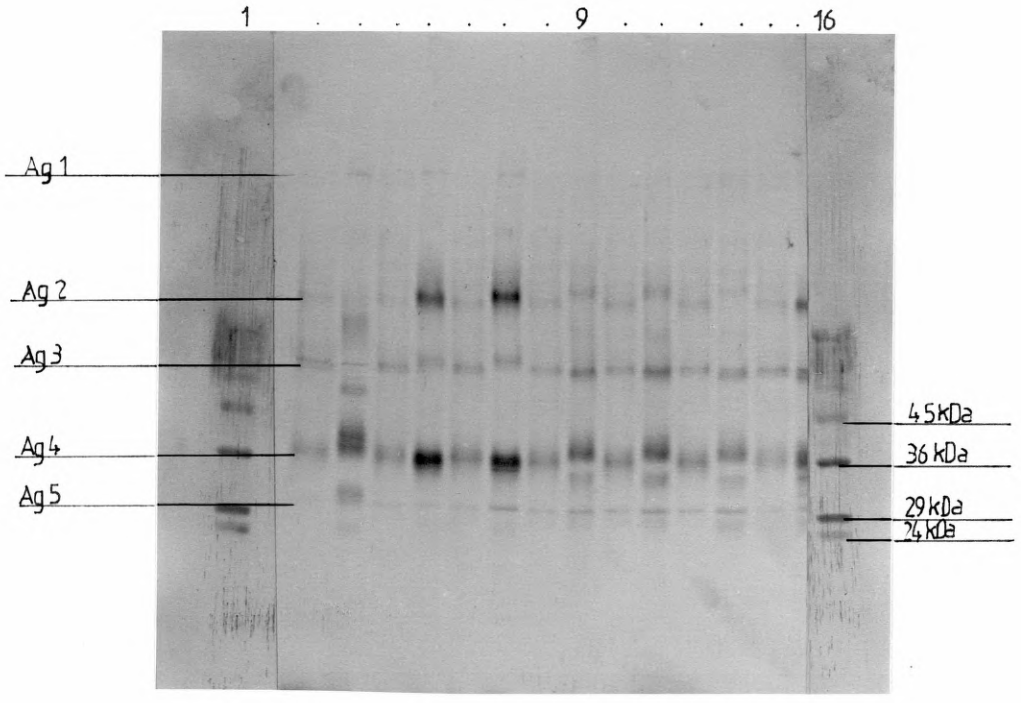


FIGURE 3.8. Western Blotting analysis of members of the *Coniophora* genus. Tracks represent: 1, 16 - MWM (Figure 3.2); 2, 4, 6, 8, 10, 12, 14 - *C. puteana* FPRL 11E; 3 - *C. arida* FPRL 411; 5 - *C. marmorata* FPRL 410; 7 - isolate DIT-U20; 9 - *C. puteana* BAM 15; 11 - *C. puteana* FPRL 11Q; 13 - *C. puteana* FPRL 11B; 15 - *C. puteana* FPRL 11A.

identity of U20 as indicated by SDS-PAGE analysis. The close similarity between FPRL 11E and 11A, confirms the higher percentage similarity value for FPRL 11A found by SDS-PAGE (Table 3.2.), the differences between these organisms and BAM 15, FPRL 11Q and 11B, may be reflected in the lower similarity indices also shown in Table 3.2.

a.

REF (11E ANTIGENS)	TEST CA	CM	U20	B15	11Q	11B	11A
1*	*	*	*				
2*							*
3*	*			*	*	*	*
4*							*
5*		*	*	*	*	*	*

b.

A	-	CP, 11A	B	-	B15, 11Q, 11B
C	-	CM, U20	D	-	CA

TABLE 3.5 - NUMERICAL ANALYSIS OF COMMON ANTIGENS IN THE GENUS *CONIOPHORA*

a. COMPARISON OF ANTIGENIC PROFILES
b. GROUPING OF ANTIGENIC PROFILES

3.4.2. ANALYSIS OF A RANGE OF FUNGI KNOWN TO INHABIT WOOD

A range of fungi known to inhabit wood were analysed by western blotting and the results are shown in Figure 3.9. Antigen bands were observed for most organisms, the major cross-reacting organisms being *H. annosum* (track 5) and *S. lacrymans* (track 13). Reaction of antiserum 88/1 with other organisms although apparent, produced fewer antigenic bands. The profile of each organism is unique and can readily be distinguished from the antigen profile of *C. puteana* (tracks 2, 9, 15). In addition, most of the basidiomycetes tested have only one antigen in common with FPRL 11E i.e. Ag 5.

3.5. SW EXTRACT: ANALYSIS OF *C. PUTEANA* AND OTHER FUNGI KNOWN TO INHABIT WOOD, BY SDS-PAGE

All fungal extracts used in this section were proteins removed in surface washings of viable mycelia (SW; the proteins removed being defined as exoproteins; 2.3.2.4.).

3.5.1. REPRODUCIBILITY OF EXOPROTEINS

An exoprotein profile was obtained using SDS-PAGE analysis. To establish if a consistent exoprotein profile could be produced for *C. puteana*, a time course experiment was undertaken and material was harvested at different stages of growth and repeatedly at the same stage (Figure 3.10.). Tracks 3-10 represent four different samples of mycelial washings after 5 days growth (sample 1 - tracks 2, 3; sample 2 - tracks 4, 5; sample 3 - tracks 6, 7; sample 4 - tracks 8, 9). There was no major alteration in the exoprotein profile in this experiment. Tracks 10 and 11

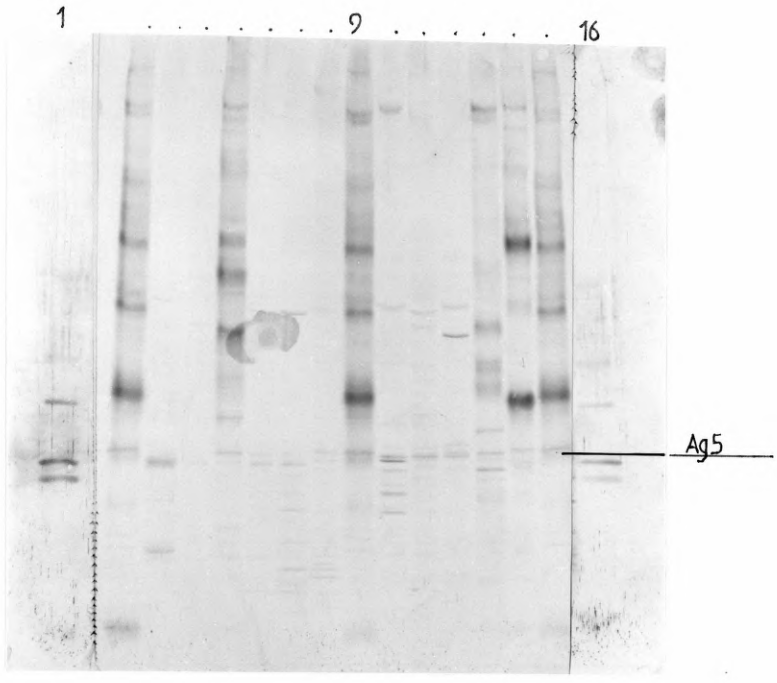


FIGURE 3.9. Western Blotting analysis of various wood inhabiting fungi. Tracks represent: 1, 16 - MWM (Figure 3.2.); 2, 9, 15 - *C. puteana* FPRL 11E; 3 - *T. harzianium* IMI 206040; 4 - *Verticillium* spp.; 5 - *H. annosum* FPRL 41E; 6 - *G. sepiarium* FPRL 10D; 7 - *G. trabeum* BAM 109; 8 - *L. sulphureus* FPRL 29; 10 - *A. xantha* FPRL 62F; 11 - *F. vaillantii* FPRL 14H; 12 - *P. placenta* FPRL 280; 13 - *S. lacrymans* FPRL 12C; 14 - isolate DIT-U20.

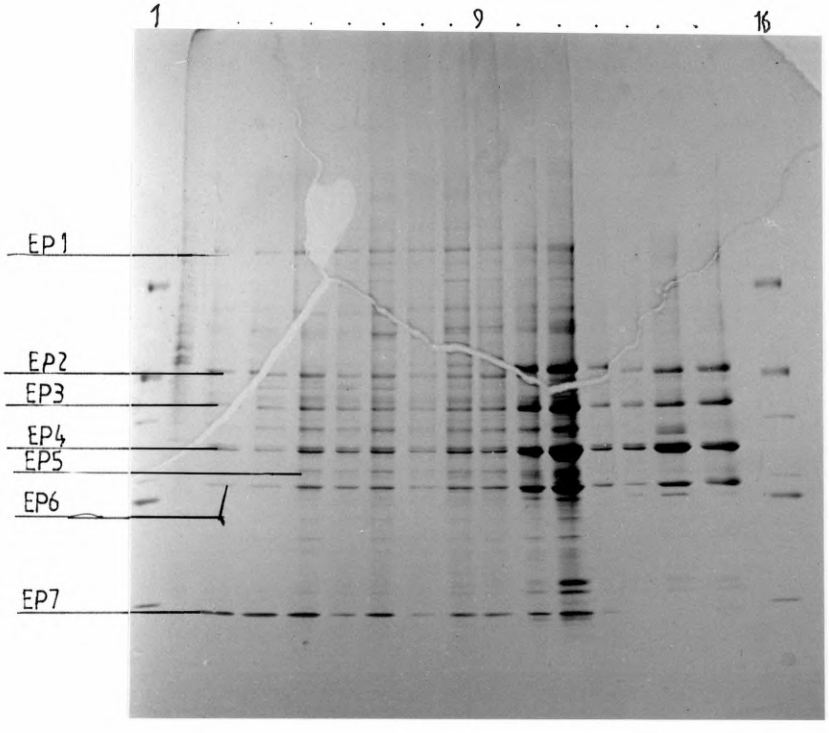


FIGURE 3.10. SDS-PAGE analysis of the reproducibility of exoproteins from *C. puteana* FPRL 11E. Tracks represent: 1, 16 - MWM (Figure 3.2.); 2, 3 - day 5 of growth, sample 1; 4, 5 - day 5 of growth, sample 2; 6, 7 - day 5 of growth, sample 3; 8, 9 - day 5 of growth, sample 4; 10, 11 - day 10 of growth; 12, 13, 14 and 15 - day 15 of growth.

represent extracts of exoproteins after 10 days of mycelial growth, tracks 12 and 15 represent growth after 15 days. Overall profiles were similar to the profiles at day 5. The greater intensity of some of the major bands is probably due to a sample concentration effect. The 7 major exoproteins evident at harvest times of day 5-10 have the molecular weights: exoprotein (EP) 1 - 75,900 Da; EP 2 - 45,700 Da; EP 3 - 38,900 Da; EP 4 - 33,100-32,400 Da (often appears as a doublet); EP 5 - 29,500 Da; EP 6 - 27,500 Da and EP 7 - 13,800 Da. Use of the exoprotein profile for identification purposes or for further studies was standardised on proteins extracted after 5-10 days growth.

3.5.2. ANALYSIS OF MEMBERS OF THE GENUS *CONIOPHORA*

To determine if the exoprotein profile obtained for *C. puteana* FPRL 11E was unique or similar to that for other members of the genus *Coniophora*, protein profiles were prepared and analysed (Figure 3.11.). The protein profiles of FPRL 11E were as obtained previously. Other strains of *C. puteana* showed notable differences specifically in the major proteins. The major proteins in common with FPRL 11E are, for FPRL 11A (track 3), EPs 2, 3, 6 and 7; for FPRL 11B (track 5), FPRL 11Q (track 7) and for BAM 15 (track 9), EPs 3, 5, 6 and 7. The remaining major proteins (EPs 1 and 4) of *C. puteana* FPRL 11E were absent. Despite these differences there still exist notable similarities between the strains of *C. puteana*, this being most evident in the less intensely stained minor bands.

Comparison of other species of *Coniophora* indicated that similarities between minor bands existed. However differences in major bands also existed, for example DIT-

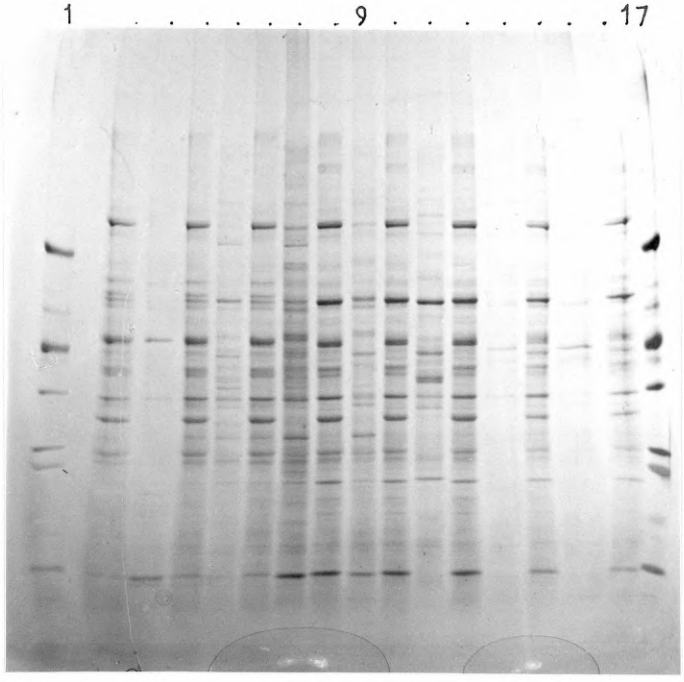


FIGURE 3.11. SDS-PAGE analysis of exoproteins of members of the *Coniophora* genus. Tracks represent: 1, 17 - MWM (Figure 3.2.); 2, 4, 6, 8, 10, 12, 14, 16 - *C. puteana* FPRL 11E; 3 - *C. puteana* FPRL 11A; 5 - *C. puteana* FPRL 11B; 7 - *C. puteana* FPRL 11Q; 9 - *C. puteana* BAM 15; 11 - isolate DIT-U20; 13 - *C. marmorata* FPRL 410; 15 - *C. arida* FPRL 411.

U20 has only 1 major protein (EP 5) in common with FPRL 11E. Analysis of other organisms is shown in Table 3.6.

TEST / REF	11A	11B	11Q	B15	U20	CM	CA
11E	4	4	4	4	1	-	-

TABLE 3.6.

COMMON MAJOR EPs OF SW EXTRACTS OF THE GENUS *CONIOPHORA* TESTED

3.5.3. ANALYSIS OF A RANGE OF FUNGI KNOWN TO INHABIT WOOD

The data in the previous section indicated that although SW preparations are more variable than WM extracts there are similarities between members of the genus *Coniophora*. The profiles for other fungi known to inhabit wood are shown in Figure 3.12. The exoproteins of these fungi can be distinguished from the reference FPRL 11E and from each other. Table 3.7. indicates the number of proteins in common with FPRL 11E. The low similarities in proteins confirms the visual analysis of results.

3.6. SW EXTRACT: INVESTIGATION OF ANTISERA 88/8

Four different exoprotein preparations of *C. puteana* were analysed and used to produce exoprotein antisera in rats (Table 3.8.). EP4 contained the greatest protein content, produced the highest antibody response and produced the most useful antisera.

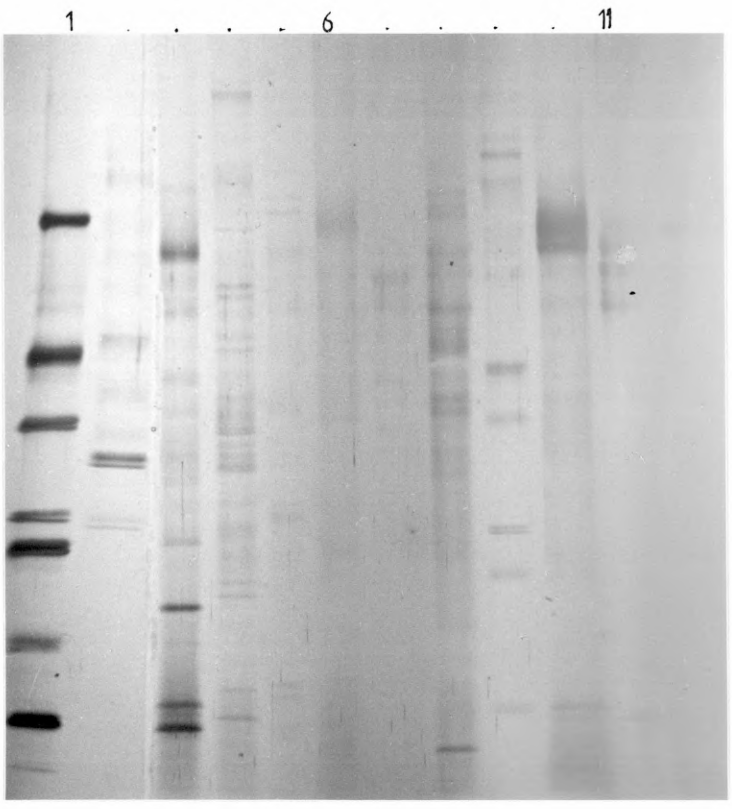


FIGURE 3.12. SDS-PAGE analysis of exoproteins from various wood inhabiting fungi. Tracks represent: 1 - MWM (Figure 3.2.); 2 - *C. puteana* FPRL 11E; 3 - *S. lacrymans* FPRL 12C; 4 - *F. vaillantii* FPRL 14H; 5 - *P. incrassata* FPRL 71; 6 - *S. sanguinolentum* FPRL 27D; 7 - *S. commune* FPRL 9; 8 - *P. panuoides* FPRL 8B; 9 - *L. sulphureus* FPRL 29; 10 - *C. versicolor* MAD 697; 11 - *P. placenta* FPRL 280.

TEST	SL	FV	PI	SS	SC	PPN	LS	CV	PP
REF									
CP	-	2	-	-	-	1	1	1	-

TABLE 3.7.

NUMBER OF COMMON PROTEINS FOR SW EXTRACTS OF FUNGI KNOWN TO INHABIT WOOD

EP SAMPLE	YIELD	PROTEIN CONTENT (µg/ml+)	NO. OF ANIMALS	A x -ve SERUM	CODE OF BEST ANTISERUM
EP1	10mg	22	4	0	88/2
EP2	180mg	37	4	1.6	88/5
EP3	3.0996g	35	4	3.5	88/7
EP4	1ml*	46	4	4.4#	88/8/C

TABLE 3.8.

ANALYSIS OF EP PREPARATIONS AND THEIR SUBSEQUENT ANTISERA

KEY

+ - Method carried out according to Biorad - Bulletin 1177EG (2.13.1.)

* - EP4 was prepared as a solution by the overlay of viable mycelia with 1ml of PBS and subsequently used as an immunogen.

- See Figure 3.13.

3.6.1. ANTIBODY DILUTION CURVE

Antibody dilution curves were constructed by testing two-fold serial dilutions of antisera 88/8/A-D against a constant antigen (EP4) concentration of 1:20 according to the method of Dewey *et al.*, (1989). A titre of 1:200 (v/v) for antiserum 88/8/C was selected for further analyses (Figure 3.13.).

3.6.2. SPECIFICITY OF ANTISERUM 88/8/C

The specificity of antiserum 88/8/C was tested against a variety of SW antigens from fungi known to inhabit wood. Reagent concentrations used were 1:20 (v/v) SW antigen preparation and 1:200 (v/v) 88/8/C. The results, which are shown in Figure 3.14., indicate the high degree of cross-reactivity of antiserum 88/8/C to a range of SW extracts. A reduction in cross-reactivity of 88/8/C when tested against white rot fungi (SC to CV) compared to brown rot fungi (CP to GS) may be apparent. *A. xantha* is the fungus which cross-reacts the most with antiserum 88/8/C.

3.7. SW EXTRACT: ANALYSIS OF *C. PUTEANA* AND OTHER WOOD INHABITING FUNGI, BY WESTERN BLOTTING

Western blotting was carried out as described in 2.11. using antiserum 88/8/C. The antigenic species reported in this section are termed exoantigens - their preparation was exactly similar to the exoproteins described in section 3.5.1.

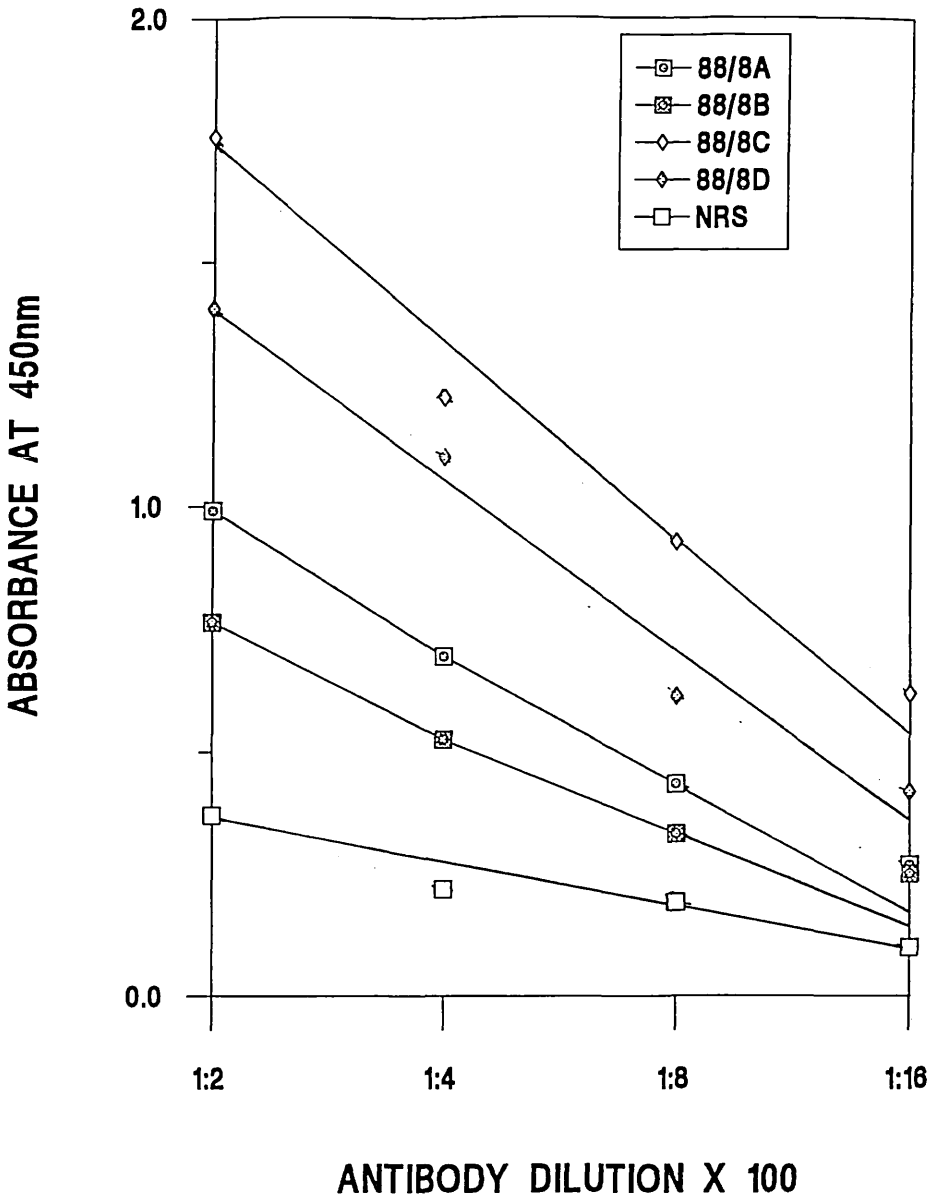


FIGURE 3.13. ANTIBODY DILUTION CURVE FOR ANTISERA 88/8

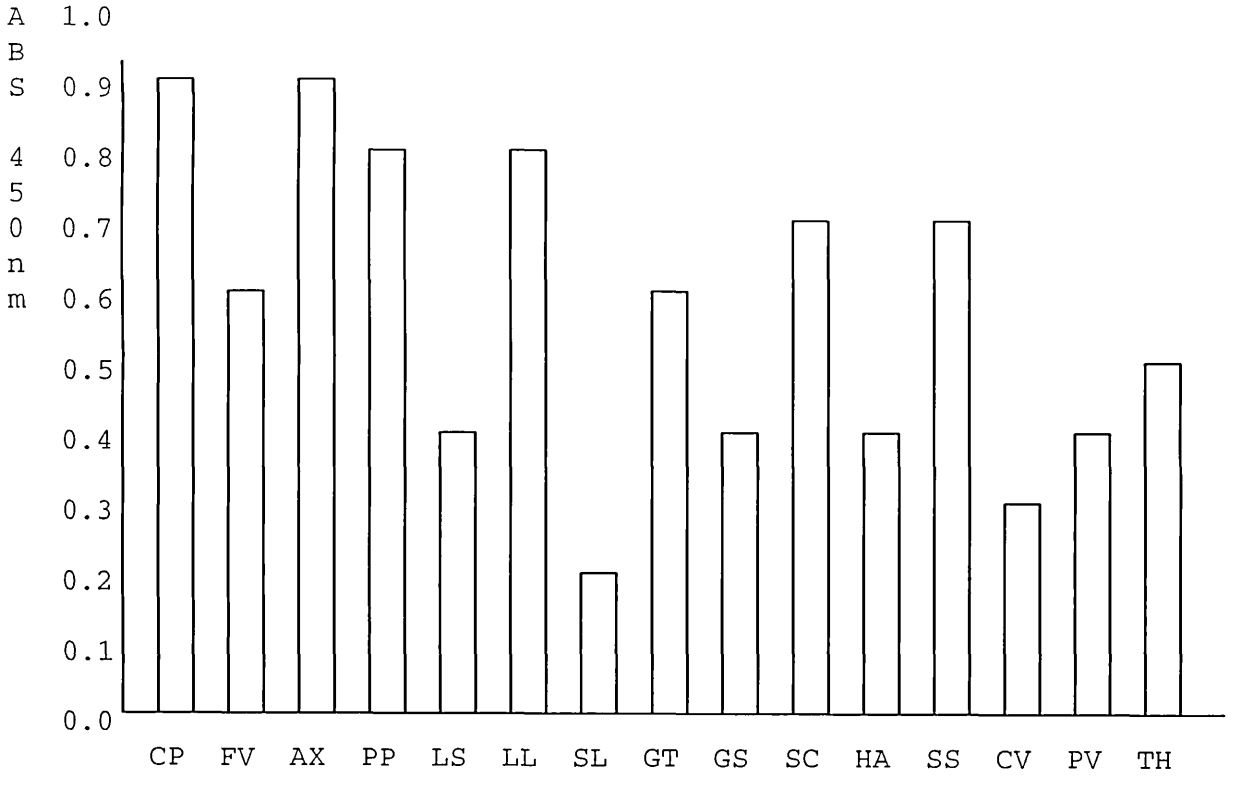


FIGURE 3.14 - CROSS-REACTIVITY OF ANTISERUM 88/8 WITH A RANGE OF FUNGI KNOWN TO INHABIT WOOD

3.7.1. REPRODUCIBILITY OF SW ANTIGEN PREPARATION

In section 3.5.1. a reproducible exoprotein profile was established by the analysis of exoproteins from different stages of the growth of *C. puteana* FPRL 11E (Figure 3.10.). Similarly the analysis of exoantigens from different stages of growth of FPRL 11E was undertaken. Figure 3.15. shows exoantigen (EA) profiles for *C. puteana* harvested at 5 days (tracks 8, 9, 10), 10 days (tracks 5, 6, 7) and 15 days (tracks 2, 3, 4) of growth. The data demonstrates the reproducibility of the antigen profiles regardless of the age of the organism. The approximate molecular weights of the 5 main EAs indicated in Figure 3.15. are 45,700 Da, 38,900 Da, 33,100-32,400 Da (often appearing as a doublet), 29,500 Da and 27,500 Da. These exoantigens correspond to 5 of the 7 major exoproteins (EPs 2, 3, 4, 5 and 6) identified during SDS-PAGE analysis in section 3.5.1., (Figure 3.10.). The presence of a faint antigen as indicated may correspond to EP1 as detailed in Figure 3.10.

3.7.2. ANALYSIS OF MEMBERS OF THE GENUS *CONIOPHORA*

To determine whether the established profile of exoantigens of *C. puteana* FPRL 11E was unique or similar to that of other members of the genus *Coniophora*, exoantigen profiles were compared and the data is shown in Figure 3.16. It was extremely difficult to produce sufficient concentrations of EPs for analysis by western blotting, hence the lack of antigens in some tracks. Nevertheless, the 5 main exoantigens (3.7.1.) for *C. puteana* FPRL 11E (even numbered tracks) are evident. In addition one antigen present in Figure 3.15. (33,100-32,400 Da), was represented as a doublet in this experiment (Figure 3.16., EA4)) and two

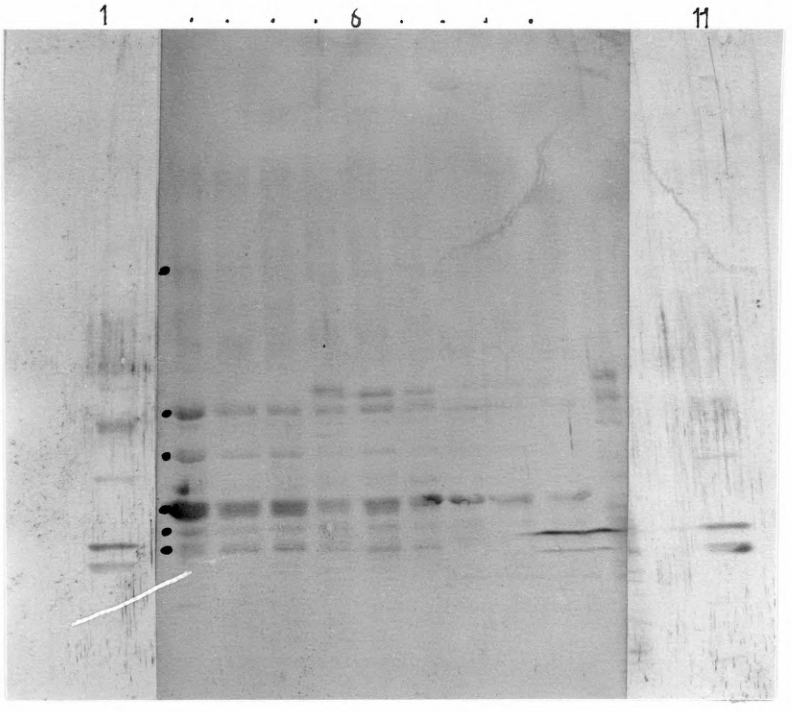


FIGURE 3.15. Western Blotting analysis of the reproducibility of exoantigens from *C. puteana* FPRL 11E. Tracks represent: 1, 11 - MWM (Figure 3.2.); 2, 3, 4 - day 15 of growth; 5, 6, 7 - day 10 of growth; 8, 9, 10 - day 5 of growth.

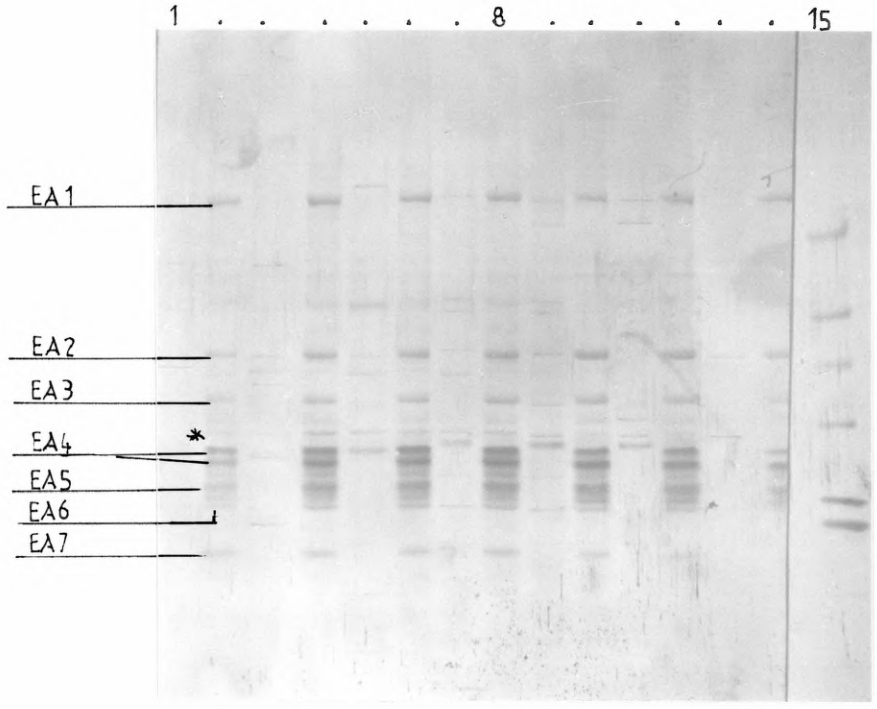


FIGURE 3.16. Western Blotting analysis of members of the genus *Coniophora*. Tracks represent: 15 - MWM (Figure 3.2.); 2, 4, 6, 8, 10, 12, 14 - *C. puteana* FPRL 11E; 1 - *C. arida* FPRL 411; 3 - *C. marmorata* FPRL 410; 5 - isolate DIT-U20; 7 - *C. puteana* BAM 15; 9 - *C. puteana* FPRL 11Q; 11 - *C. puteana* FPRL 11B; 13 - *C. puteana* FPRL 11A.

minor components appeared more strongly stained (EA1 and EA7). In total therefore 7 antigens can be recognised; EA 1 - 75,900 Da; EA 2 - 45,700 Da; EA 3 - 38,900 Da; EA 4 - 33,100 and 32,400 Da doublet; EA 5 - 29,500 Da, EA 6 - 27,500 Da and EA 7 - 21,900 Da. EAs 1-6 correspond to the major proteins which were observed when *C. puteana* FPRL 11E was analysed by SDS-PAGE (section 3.5.1., Figure 3.10.), whilst EA 7 does not relate to any EP evident in Figure 3.10. although it may be faintly obvious in Figure 3.15., as indicated.

Initial observations suggest that there is a diversity of EA throughout the *Coniophora* genus, however, there are similarities which allow a relationship between the profiles to become established. The presence of EAs 1, 2 and 6 of *C. puteana* FPRL 11E within the *C. puteana* species distinguishes the species from others within the genus. Whilst the presence of EA 2 and EA* (a minor antigen) distinguish the *Coniophora* genus from all other organisms (see later). Although DIT-U20 resembles *C. marmorata* in other analyses, the antigenic profiles of *C. marmorata* and DIT-U20 are not identical.

3.7.3. ANALYSIS OF A RANGE OF FUNGI KNOWN TO INHABIT WOOD

Exoantigen extracts of a range of fungi known to inhabit wood were analysed by western blotting and the results are shown in Figure 3.17. Antiserum 88/8 cross-reacted with most organisms in this analysis, producing unique antigen bands which could be clearly distinguished from the antigens of *C. puteana* FPRL 11E (track 15).

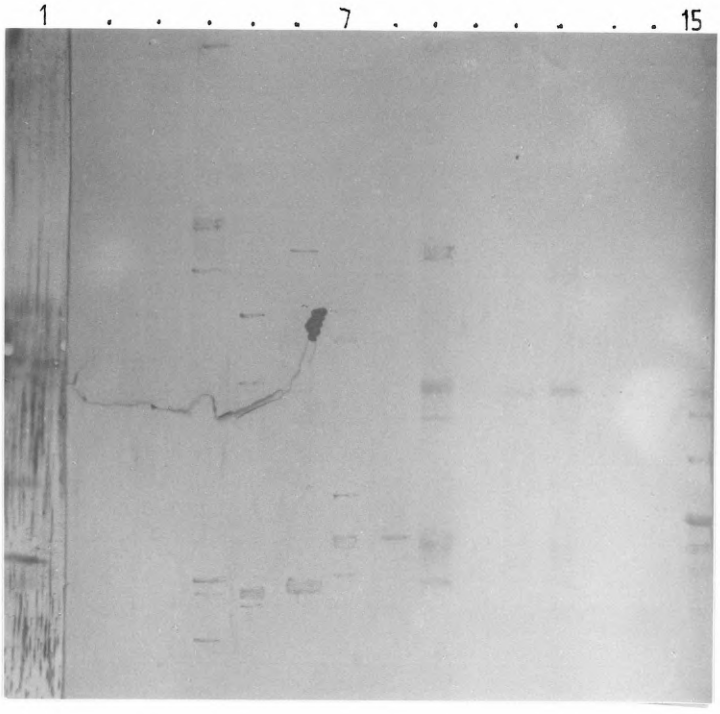


FIGURE 3.17. Western Blotting analysis of exantigens from various wood inhabiting fungi. Tracks represent: 1 - MWM (Figure 3.2.); 2 - *P. placenta* FPRL 280; 3 - *C. versicolor* MAD 697; 4 - *L. sulphureus* FPRL 29; 5 - *P. panuoides* FPRL 8B; 6 - *S. sanguinolentum* FPRL 27D; 7 - *P. incrassata* FPRL 71; 8 - *F. vaillantii* FPRL 14H; 9 - *S. lacrymans* FPRL 12C; 10 - *G. trabeum*; 11 - *G. sepiarium*; 12 - *H. annosum*; 13 - *Verticillium* spp.; 14 - *T. harzianium*; 15 - *C. puteana* FPRL 11E.

3.8. DISCUSSION

The research reported in this chapter, can be divided into the study of the application of two techniques, viz., SDS-PAGE and western blotting to the analysis of wood decay basidiomycetes. The potential of both techniques for the identification of *C. puteana* and other fungal organisms will be discussed. The various extracts and antisera produced will also be considered: the relevance of each in the identification process being highlighted.

3.8.1. SDS-PAGE

SDS-PAGE has for the past 22 years been used as a tool for the identification and analysis of various fungal types, particularly fungi pathogenic to man, e.g. the classification of *C. albicans* (Lee *et al.*, 1986). This method of identification, based on the finding that fungi within a genus have related protein profiles, has recently been investigated by researchers studying wood decay fungi. In particular isolates of *S. lacrymans* have been shown to have substantial protein similarities using a standard protein extract (WM) (Schmidt and Kebernik, 1989; Palfreyman *et al.*, 1991a). That the proteins of WM extracts of *C. puteana* have a high degree of similarity when compared to other members of the genus *Coniophora* is confirmed in this current study (M^{c}Dowell *et al.*, 1992a and b).

In addition to similarities within the *Coniophora* genus, inter-species differentiation is also evident which allows *C. puteana* and related organisms to be distinguished from all other fungal species. Inter-species analysis of other

basidiomycetes using SDS-PAGE profiles has also allowed the differentiation of *S. lacrymans* and *H. annosum* from a range of fungi known to inhabit wood (Vigrow *et al.*, 1989; Galbraith, personal communication). Further, intra-species differentiation of *Coniophora* organisms as well as inter-species differentiation of *C. puteana* is evident from the current studies and thus allows the distinguishment of individual organisms within the *Coniophora* genus. The differentiation of *Coniophora* organisms is confirmed by the fact that similarity indices for strains of *C. puteana* were never 100%. Contrasting results have been reported for strains of *S. lacrymans*, the similarity indices of which were often 100% (Palfreyman *et al.*, 1991a). The intra-species variation of the protein profiles of *C. puteana* suggests that certainly *C. puteana* and possibly other members of the *Coniophora* genus have greater phenotypic and possibly genetic variability than *S. lacrymans*. This variation within the *C. puteana* species is not so great as to interfere with the recognition of a general protein profile of *C. puteana* for identification purposes. Studies reported elsewhere for fungi not associated with wood decay also suggest that the differentiation of organisms by SDS-PAGE is possible, for example, the distinction of *Gaeumannomyces graminis* var. *tritici* from *Phialophora* spp. (Mass *et al.*, 1990).

To date, studies of methodologies for the identification of fungi by SDS-PAGE have generally only used extracts of whole mycelia to produce standard profiles (Milton *et al.*, 1971; Hansen *et al.*, 1988; Schmidt and Kebernik, 1989; Maas *et al.*, 1990; Maiden and Tanner, 1991). Researchers in the medical field, such as Kaufman and Standard (1987) have however based diagnosis of fungal disease and

identification of the infecting organism on the production of antibodies to soluble proteins (exoantigens), specific to individual organisms and which are easily extracted. These exoantigens have proven valuable for the immunoidentification of fungal pathogens, for example, *Histoplasma capsulatum* (Standard and Kaufman, 1976). The intention of the studies in this chapter was to determine if wood decay fungi could be identified by SDS-PAGE using an analogous method of sample preparation to that used by Kaufman and Standard (1987) but examining the proteins (exoproteins) as opposed to antigens. In addition, the use of exoproteins for the production of a more specific antiserum was examined (see later).

The results presented indicate that identification of *C. puteana* and other members of the genus *Coniophora* is feasible using exoproteins, since a profile which is unique to the genus was produced. However a greater degree of variation of exoprotein profiles exists between members of the genus *Coniophora* and particularly within the species *C. puteana*, compared to the variation which was perceived for WM extracts of such organisms. These results suggest that the surface proteins of the mycelia of *C. puteana* may be more diverse than the internal proteins examined by WM extraction. In addition, some of the exoproteins appear to be unique in comparison to the proteins of the WM extract i.e. EPs 2, 3, 6 and 7 (Figure 3.10.) whilst the remaining EPs (1, 4 and 5) may be similar to protein species within the WM extract. These unique EPs may have specific functions which will be discussed in chapter 4. Despite the differences in profiles within the *Coniophora* genus, identification is feasible using this more variable extract. It should be noted that further intra-species

differentiation within the genus could be facilitated by the use of this extract in conjunction with the use of the standard extract.

3.8.2. WESTERN BLOTTING

The analysis of *Coniophora* organisms by western blotting using polyclonal antisera produced two specific antigen profiles: one for WM extracts and one for SW extracts, with their corresponding antiserum. Five antigens were evident for the WM extracts, whilst 7 exoantigens (EA) were exhibited for the SW extracts. EAs 1, 2, 4, 6 and 7 are unique to the SW extracts, whilst EAs 3 and 5 may be in the same molecular weight range as antigens of the WM extract. These unique EAs, like the unique EPs may have specific functions and will be further discussed in chapter 4. The WM and SW antisera were also shown to be cross-reactive towards other wood decay fungi. Since western blotting allows the analysis of individual antigens probed by polyclonal antisera, wood decay fungi were analysed to determine if the antigens detected were the same or different to the antigens of *Coniophora* organisms. Decay fungi were shown to produce antigens which were different to those present within the *Coniophora* profiles. Antigen profiles can therefore be used to identify individual organisms.

It is generally recognised in all areas of immunology, that polyclonal antisera will show some degree of cross-reactivity. In wood decay studies several antisera have been developed to various decay fungi, which also react with the antigens of many other fungi. The high degree of cross-reactivity of an antiserum to WM extracts of *Poria*

placenta using EIA was described by Goodell and Jellison (1986). Similarly studies of WM extracts of the organisms, *S. lacrymans* and *Lentinus lepideus* by a variety of methods suggested that antisera to all organisms were highly cross-reactive (Palfreyman *et al.*, 1988a; Glancy *et al.*, 1989; Vigrow *et al.*, 1991b; Vigrow, 1992). Since these antisera are not specific for the immunogen, use of the sera as general immune reagents for possible identification methods can be exploited.

Recently this technique has been utilised as a tool for the identification of wood decay fungi based on the antigen profiles detected by polyclonal antisera, e.g. the identification of *S. lacrymans* (Vigrow *et al.*, 1991a and b). The analysis of the antigen profiles of WM extracts of strains of *S. lacrymans* probed by a cross-reacting polyclonal antiserum indicated that the profiles were similar (Palfreyman *et al.*, 1988), a result confirmed in a later, more complete study (Vigrow *et al.*, 1991b). In 1989, Glancy *et al.*, reported analogous results for *L. lepideus* antigens. Current studies indicate that *C. puteana* antigens show similar consistency and support the potential of western blotting as an instrument for the identification of both *C. puteana* and other fungi known to inhabit wood. Similar results have been reported elsewhere, e.g. the comparative immunochemical analysis of 5 *Thermoactinomyces* strains indicated the presence of common antigens within the species (Ylonen *et al.*, 1989) and the analysis of strains of *Aspergillus fumigatus* using polyclonal antisera revealed antigenic similarities between strains of the organism (Burnie *et al.*, 1989; Hearn *et al.*, 1990).

In addition to the similarities within the antigen profiles of the different strains of *C. puteana*, intra-species variation of *C. puteana* was also apparent, since 2 antigen profiles were evident (A and B, 3.4.1.). The other species of *Coniophora* tested, *C. arida* and *C. marmorata*, exhibited further variation in their antigen profiles indicating that differentiation of species is possible by western blotting, a conclusion drawn from SDS-PAGE which required confirmation by similarity indices. Re-analysis of the SDS-PAGE profile in the light of the antigen analysis reveals possible differences between FPRL 11E, FPRL 11A and the other organisms (see Table 3.2.(i); the similarity index value for FPRL 11A in comparison to FPRL 11E is 83%, whilst the mean similarity index value for FPRL 11B, FPRL 11Q and BAM 15 in comparison to FPRL 11E is 73%). Together these results indicate that perhaps the organisms, FPRL 11Q, 11B and BAM 15 may have been mis-classified.

The identification of different antigens and possibly protein profiles within the species *C. puteana* is consistent with the flux that is apparent in the taxonomic and nomenclature relationships between different basidiomycetes. Re-classification of organisms is frequent, e.g. the incorporation of *Poria incrassata* into the genus *Serpula*; and the identification of specific organisms is sometimes incorrect, e.g. a strain of *S. lacrymans* which has recently been re-classified as a strain of *S. himantioides* (Vigrow *et al.*, 1991). In general the molecular studies reported in this chapter emphasise the relevance of western blotting as a tool for identification of wood decay fungi. In addition, western blotting may contribute to taxonomic studies by the production of simple antigen profiles which may alleviate the problems

encountered in the naming and classification of wood decay fungi.

Studies on exoantigens of medically important fungi by numerous researchers indicate that it is possible to identify fungi based on the high degree of specificity of their exoantigens (Sekhon *et al.*, 1986; Cox and Britt, 1986; Yangco *et al.*, 1986; Kaufman and Standard, 1987). The exoantigen test of double diffusion used by these researchers allowed the identification of specific antigens by the precipitation of the exoantigen with polyclonal antisera and the recognition of this antigen-antibody precipitate by the production of a line of identity with a reference antigen-antibody precipitate. Since in this current study, the *Coniophora* genus has been demonstrated as having a common exoprotein profile which is different from that of other fungi known to inhabit wood, an antiserum against exoproteins was produced in an attempt to provide a more specific polyclonal antisera to *C. puteana* for identification purposes. The exoantigen antiserum produced however, was also cross-reactive. Such cross-reactivity has been found for antisera produced against exoproteins of *L. edodes*, *Tyromyces palustris*, *C. versicolor* and *P. placenta* (Kim *et al.*, 1991) and the resultant cross-reactivity varies from organism to organism.

Due to the nature of the *C. puteana* exoantigen antiserum to cross-react with other basidiomycetes, it was possible to exploit the antiserum as a reagent for identification purposes using western blotting. Analysis by western blotting using this antiserum detected antigens for most decay fungi tested; antigens which were unique to each

organism and which could be distinguished from a set of common antigens of the *Coniophora* genus. In addition, like the WM extracts, differentiation of members of the genus was possible, although there was a greater diversity of SW antigens within the genus in comparison to the substantially similar WM antigens. Nevertheless, a recognisable exoantigen profile was evident for *Coniophora* organisms which could easily be differentiated from other fungi known to inhabit wood.

Like the exoantigen technique previously described, western blotting using an exoantigen antiserum for *C. puteana* produces specific exoantigens for *C. puteana* which can be distinguished from those of other fungi. An advantage of western blotting analysis however is the ability of the technique to show antigenic similarities between organisms which allows a profile for the genus *Coniophora* to become established. In addition western blotting is more favourable for identification since the analysis of the more complex WM extract is also possible, whereas the interpretation of double diffusion using such an extract is extremely complex.

A final feature of the exoantigens relates to their correlation with the proteins of the SW extract. Seven antigens of the SW extract shared the same molecular weights as the 7 major proteins exhibited by SDS-PAGE. There were only 5 major WM antigens detected using the WM antiserum, whilst the proteins of this extract were numerous. This suggests that compared to the proteins of the WM extract, EPs are highly immunogenic. In addition, the production of the *C. puteana* antiserum against EPs of this organism did not require the use of an adjuvant. EPs

from other organisms, used for the immuno-diagnosis of diseases have also been described as highly immunogenic and have consequently been utilised for the production of specific monoclonal antibodies (Kaufman and Standard, 1987). The exoantigens described in this chapter could therefore be useful for the production of specific monoclonal antibodies for the detection of *C. puteana*.

3.8.3. CONCLUSIONS

Identification of *C. puteana* is possible by visual interpretation of SDS-PAGE gels and confirmation of identification can be obtained by the calculation of similarity indices. Western blotting is relevant as an adjunct to SDS-PAGE to further confirm identity and to amplify differences between organisms hitherto undetected by SDS-PAGE. The interpretation of the profiles produced by western blotting is much simpler and therefore this technique may be more useful as a method of identification than SDS-PAGE. The use of different extracts of *C. puteana* highlighted the differences in the identification ability of both techniques when various extracts were used. The standard WM extract is however the most reliable starting point for initial identification. If further analysis of a genus is required, the exoprotein extract will be useful since further differentiation between related organisms is possible. In addition, information on the antigenic nature of the organism is obtained as well as information on the immunogenicity of the antigen, which is relevant for the production of specific probes. In conclusion, the methods described can be used to identify *C. puteana* and other fungal organisms, provided a reference strain is available.

CHAPTER 4

MOLECULAR AND IMMUNOLOGICAL CHARACTERISATION OF *C. PUTEANA*

4.1. INTRODUCTION

One of the aims of the research described in this thesis was to develop simple identification and detection systems for the wet rot organism *C. puteana*. Chapter 3 detailed systems for the preliminary identification of *C. puteana* and other fungi known to inhabit wood. These systems however were developed by the examination of *C. puteana* under cultural conditions that may not reflect the growth of this organism in nature. Consequently an investigation of the effect of media types which are more similar to wood, on the growth, and protein and antigen profiles of WM and EP extracts of *C. puteana* was carried out. Also studied was the effect of culture age on these profiles. In addition an investigation of possible specific differences within the profiles produced from different parts of *C. puteana* mycelia was undertaken to provide information on the degree of variability of specific antigens. The performance of the immunological reagents described in chapter 3, was also assessed by reference to an antiserum raised against a growing tip (GT) extract of *C. puteana*.

4.2. THE PRODUCTION AND ANALYSIS OF GT ANTISERA

Studies on *S. lacrymans* have indicated differences in antigenic profiles in different regions of agar cultures (Vigrow *et al.*, 1991). To investigate the existence of such differences for *C. puteana*, extracts of similar regions were analysed. In addition to an examination of the extracts with the immunological reagents available, antisera against GT extracts were produced to investigate the possible presence of growing tip antigens, which may be useful markers for actively growing *C. puteana* in wood and

other substrates. The titres of these antisera are shown in Figure 4.1. Antiserum 90/2/C (GTC) was selected for further analysis and used at a dilution of 1:400 (v/v). Cross-reactivity with a range of fungi known to inhabit wood was tested (Figure 4.2.) and the antiserum was shown to cross-react mainly with brown rot fungi, with the exception of *P. variotii*. A minor degree of cross-reactivity was observed with the other fungi tested. No cross-reactivity for this antiserum was observed with *S. lacrymans*.

4.3. PRODUCTION OF STANDARD ANTIGEN PROFILES FOR ANTISERA 88/1, 88/8 AND 90/2

The characterisation of *C. puteana* antigens from WM, EP and GT extracts was undertaken using the antiserum 88/1, antiserum 88/8 (against EP extract) and antiserum 90/2 (against GT extract) using western blotting (Figure 4.3.). The purpose of this analysis was to compare standard profiles produced by homologous antiserum with other combinations of antigen/antibody. To aid in the interpretation of results, the graphical representation shown in Figure 4.4. was produced.

The profile shown in track 10 is the same as the standard WM profile (Figure 3.8.), as expected; Ags 1-5; 136,500 Da; 98,900-92,300 Da; 68,400-65,300 Da; 39,500-37,000 Da and 32,000 Da, are highlighted. The GT and EA profiles (track 8 and 9, respectively) contained similar antigens but the intensity of some bands were markedly different in the latter profile. The antigen profiles produced by EA antiserum 88/8 (tracks 5, 6, 7) were essentially similar to the standard profile (Figure 3.16.); EA 2 - 45,700 Da; EA 3 - 38,900 Da; EA 4 - 33,100 Da, 32,400 Da doublet and EA 5 -

other substrates. The titres of these antisera are shown in Figure 4.1. Antiserum 90/2/C (GTC) was selected for further analysis and used at a dilution of 1:400 (v/v). Cross-reactivity with a range of fungi known to inhabit wood was tested (Figure 4.2.) and the antiserum was shown to cross-react mainly with brown rot fungi, with the exception of *P. variotti*. A minor degree of cross-reactivity was observed with the other fungi tested. No cross-reactivity for this antiserum was observed with *S. lacrymans*.

4.3. PRODUCTION OF STANDARD ANTIGEN PROFILES FOR ANTISERA 88/1, 88/8 AND 90/2

The characterisation of *C. puteana* antigens from WM, EP and GT extracts was undertaken using the antiserum 88/1, antiserum 88/8 (against EP extract) and antiserum 90/2 (against GT extract) using western blotting (Figure 4.3.). The purpose of this analysis was to compare standard profiles produced by homologous antiserum with other combinations of antigen/antibody. To aid in the interpretation of results, the graphical representation shown in Figure 4.4. was produced.

The profile shown in track 10 is the same as the standard WM profile (Figure 3.8.), as expected; Ags 1-5; 136,500 Da; 98,900-92,300 Da; 68,400-65,300 Da; 39,500-37,000 Da and 32,000 Da, are highlighted. The GT and EA profiles (track 8 and 9, respectively) contained similar antigens but the intensity of some bands were markedly different in the latter profile. The antigen profiles produced by EA antiserum 88/8 (tracks 5, 6, 7) were essentially similar to the standard profile (Figure 3.16.); EA 2 - 45,700 Da; EA 3 - 38,900 Da; EA 4 - 33,100 Da, 32,400 Da doublet and EA 5 -

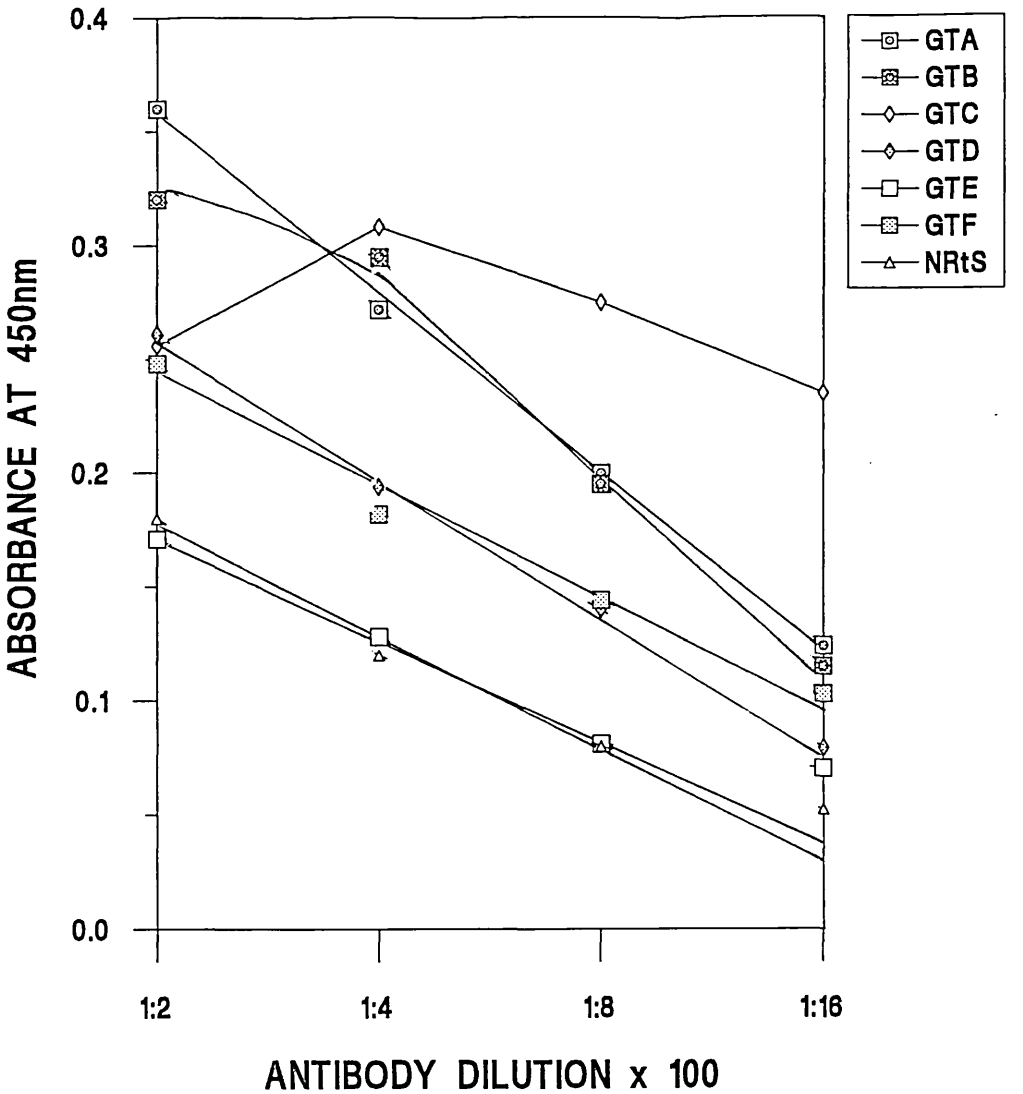


FIGURE 4.1. ANTIBODY DILUTION CURVE FOR ANTISERA 90/2

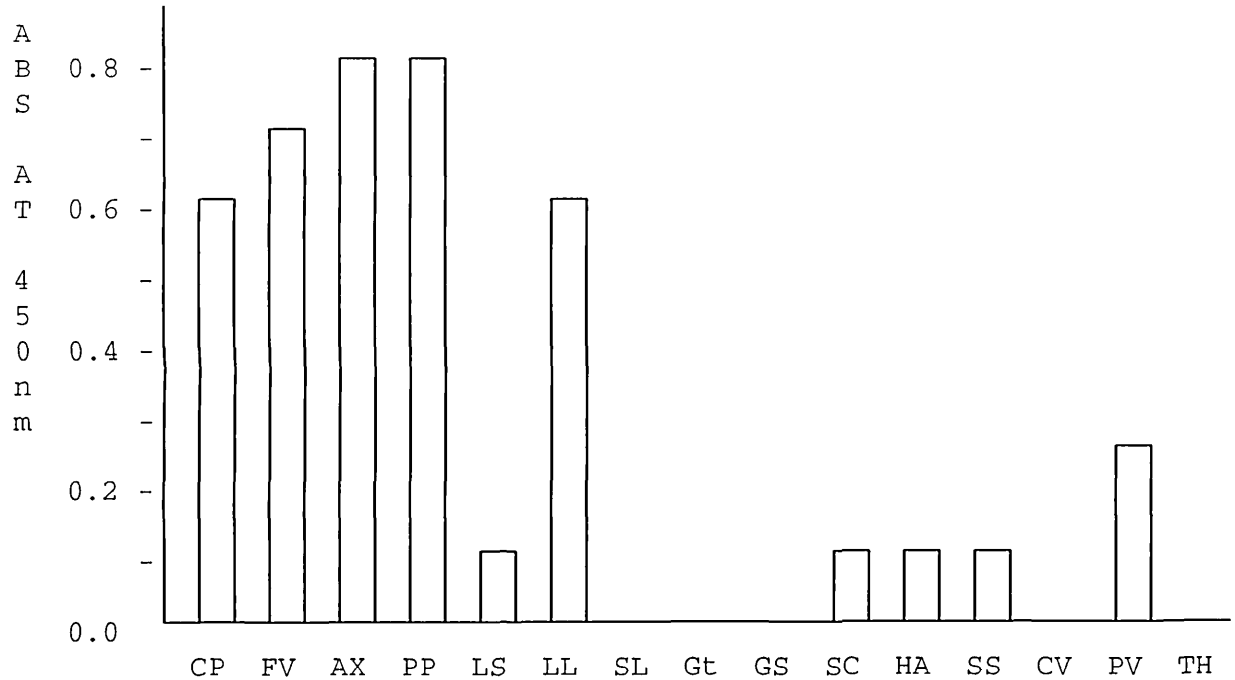


FIGURE 4.2. CROSS-REACTIVITY OF ANTISERUM 90/2/C

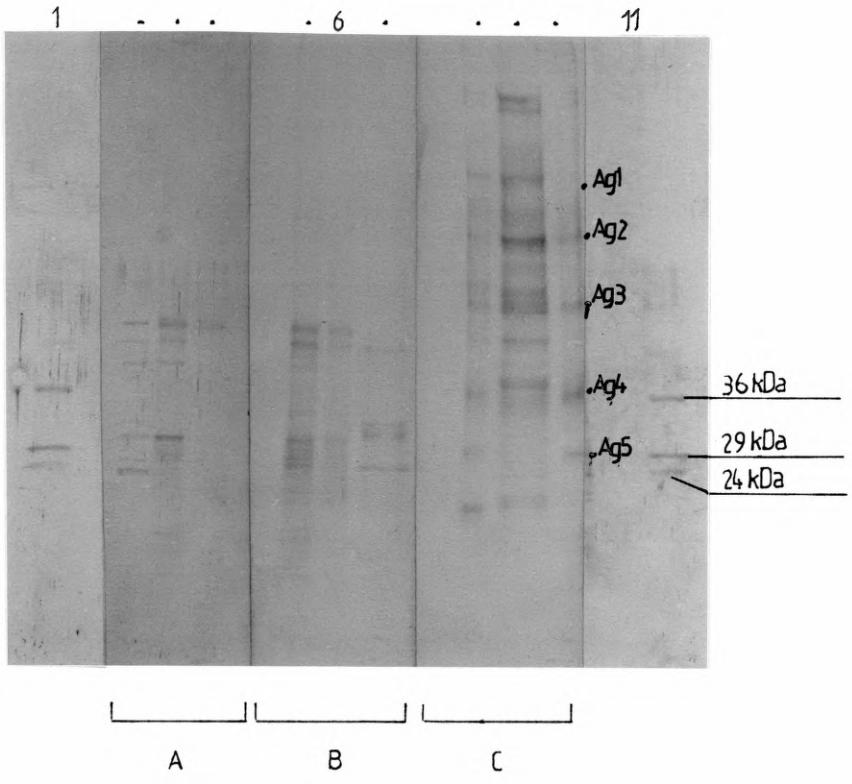


FIGURE 4.3. Western blotting analysis of protein extracts of *C. puteana* using antisera 90/2 (A), 88/8 (B) and 88/1 (C). Tracks represent: 1, 11 - Standard molecular weight markers (MWM); 2, 7, 9 - EA; 3, 5, 10 - WM; 4, 6, 8 - GT. Standard profiles are: 4 - GT extract/antiserum 90/2; 7 - EA extract/antiserum 88/8; 10 - WM extract/antiserum 88/1. In this and all subsequent figures the molecular weight markers used were of the following sizes, 14,200, 20,100, 24,000, 29,000, 36,000, 45,000 and 66,000 Daltons.

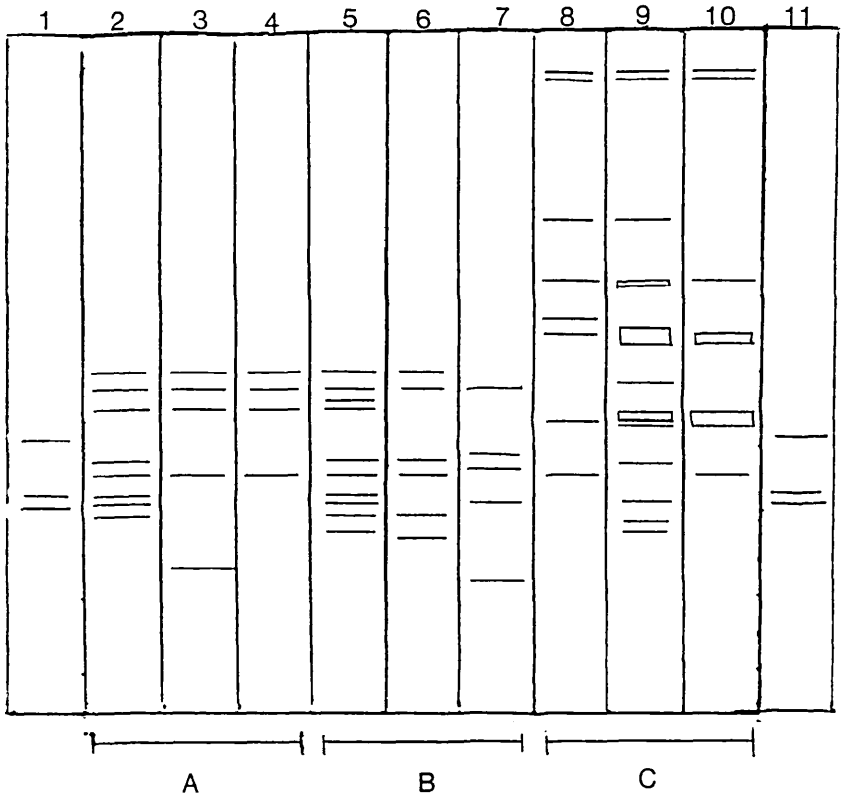


FIGURE 4.4. Schematic diagram of antigen profiles of various extracts of *C. puteana* when probed with 3 antisera, A - 90/2, B - 88/8 and C - 88/1. Tracks represent: 2, 7, 9 - EA; 3, 5, 10 - WM; 4, 6, 8 - GT. Standard profiles are: 4 - GT extract/antiserum 90/2; 7 - EA extract/antiserum 88/8; 10 - WM extract/antiserum 88/1.

29,500 Da). The antigen profiles of the WM and GT extracts (tracks 5 and 6, respectively), exhibited some variation from the profile of the EA extract (track 7). The antigen profiles produced by the extracts when analysed using the GT antiserum 90/2 (tracks 2, 3, 4) and EA antiserum 88/8 (tracks 5, 6, 7) share some common antigen bands, but differences between homologous extracts are apparent.

4.4. THE EFFECT OF VARIOUS GROWTH PARAMETERS ON THE MORPHOLOGY OF MYCELIA AND PROTEIN/ANTIGEN PROFILES OF *C. PUTEANA*

The direct analysis of mycelium growing in wood is difficult, since mycelium is not always visible. In order to analyse the morphology of *C. puteana* mycelia under "natural" conditions, the agar based substrate was altered to mimic the condition of wood. The consequent protein profiles of various extracts of the organism were analysed by SDS-PAGE in comparison to the standard profile, to determine possible changes within the profiles. Western blotting analysis of extracts of *C. puteana* using the antisera described in section 4.3. was also undertaken to analyse possible changes in the antigen profiles of *C. puteana*.

4.4.1. MORPHOLOGICAL ANALYSIS

C. puteana was cultured on 5% MXB (standard preparation; liquid medium), 2% A/5% MX (agar medium), 2% A/5% C (cellulose medium), and 2% A/5% S (sawdust medium) (Figures 4.5. and 4.6.). The morphology and growth of *C. puteana* was affected by change of substrate. The mycelium of *C. puteana*, when grown on liquid medium, was cream in colour,

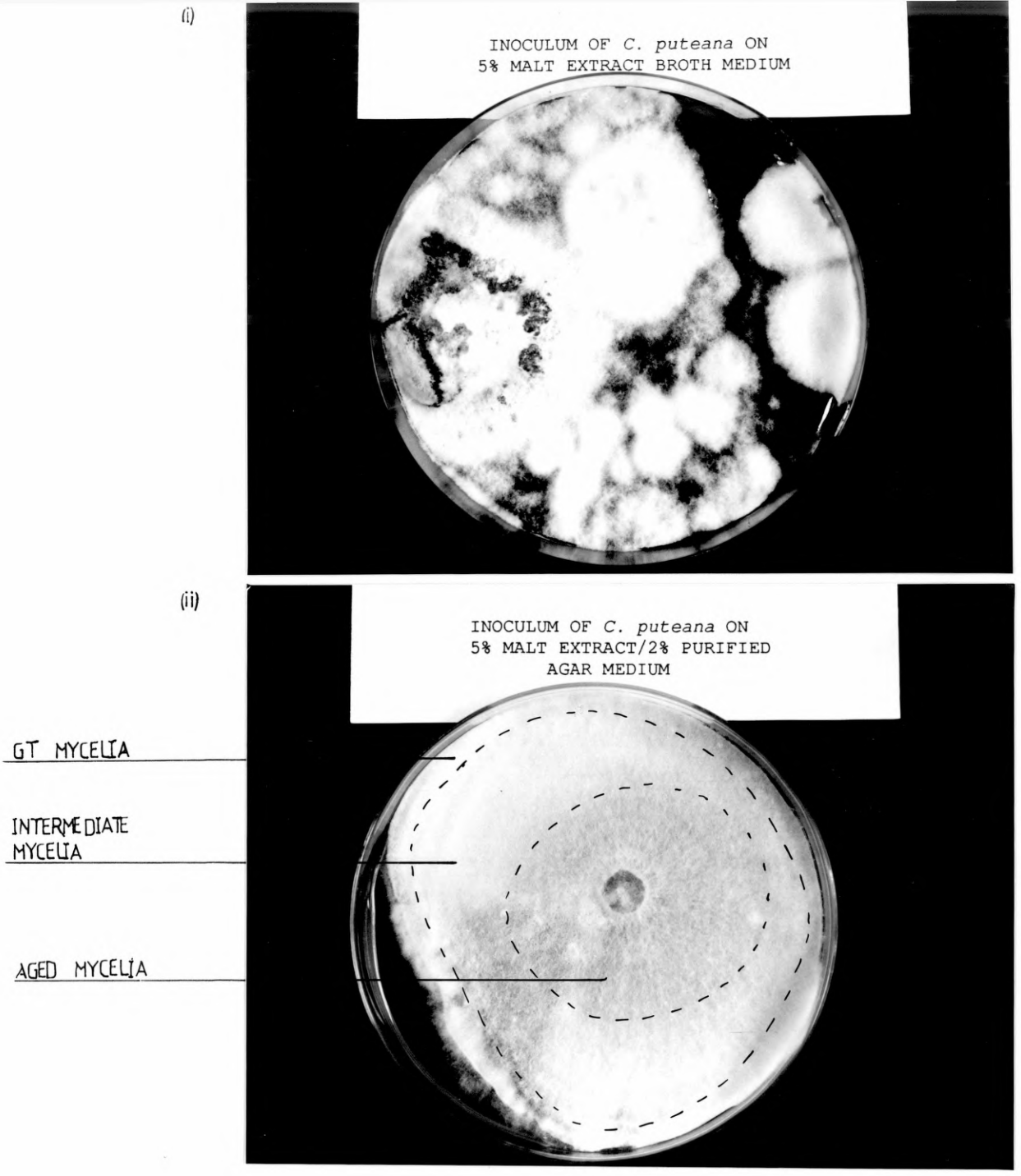


FIGURE 4.5. *C. puteana* FPRL 11E cultured on (i) 5% MXB and (ii) 2% A/5% MX.

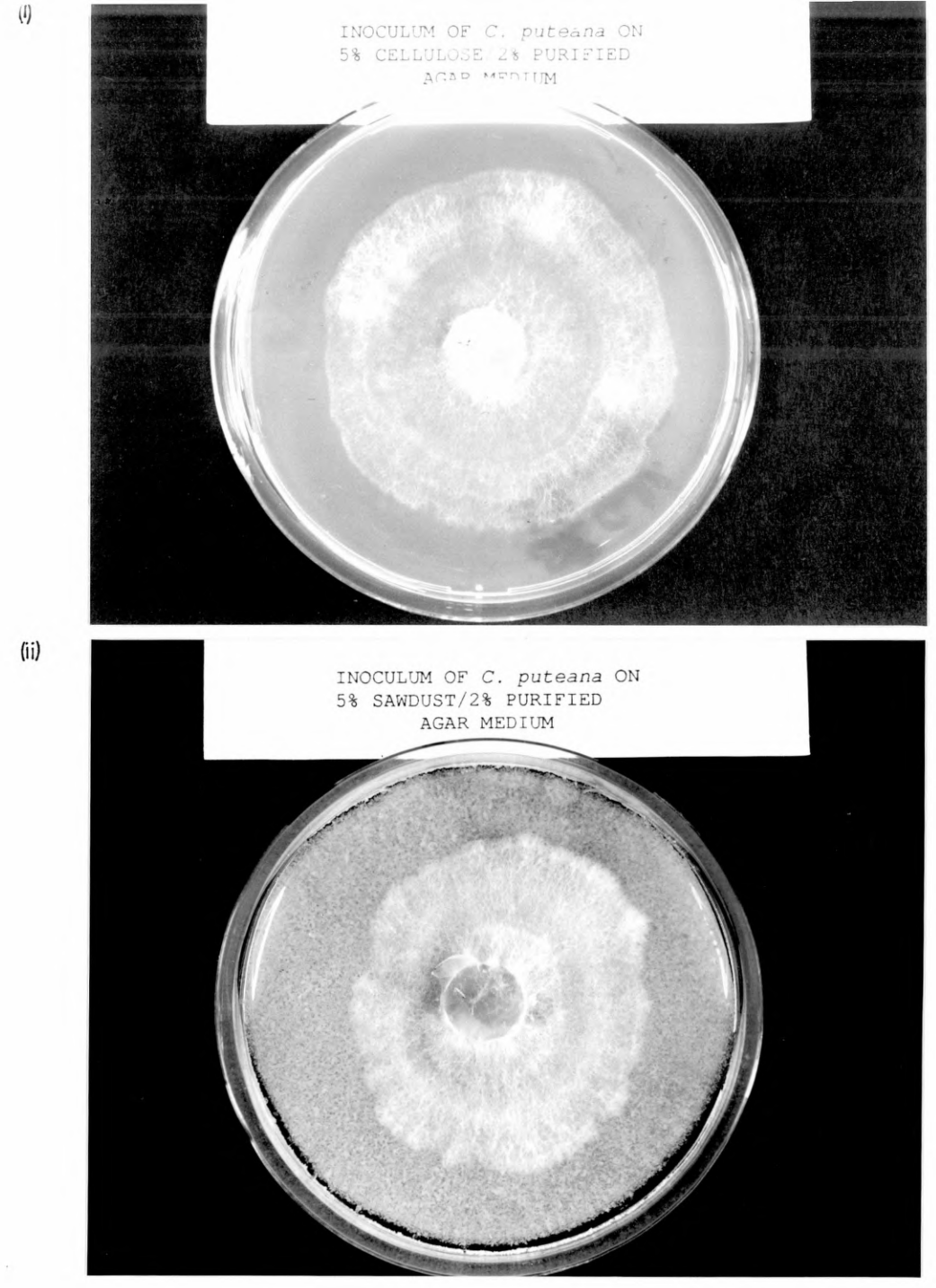


FIGURE 4.6. *C. puteana* FPRL 11E cultured on (i) 2% A/5% C and (ii) 2% A/5% S.

often associated with surface secretions of yellow/brown liquid, typically floccose and raised about 3mm. A similar type of mycelia was evident on the agar medium except that growth was much less random, with the production of a continuous hyphal front. Three distinct regions were evident as indicated, i) the growing hyphal tip (GT), which is composed of typically floccose material; ii) intermediate mycelia and, iii) aged, more sparse mycelia which lacked aerial growth. However, when *C. puteana* was grown on cellulose or sawdust medium, the organism was clearly stressed, producing thin sparse appressed mycelia. Floccose mycelia was only evident at the advancing edge of the colony. *C. puteana* grown on sawdust medium appeared to degrade the media directly below the mycelia (not shown in Figure), and grew relatively slowly over the medium. The radial growth rate of *C. puteana* grown on liquid or agar medium in a 90mm petri dish was an average 6mm/day. On sawdust or cellulose medium, the growth rate was reduced to 4.5mm/day.

4.4.2. MOLECULAR ANALYSIS OF *C. PUTEANA* GROWN ON DIFFERENT SUBSTRATES

WM and EP extracts were prepared every 2 days after the subculture of *C. puteana*. Extracts were analysed by SDS-PAGE and Figures 4.7. and 4.8. show the results obtained for WM and EP respectively. From Figure 4.7., it can be seen that the profiles produced when *C. puteana* was grown on agar (tracks 3, 5, 7, 9, 11, 13) showed substantial similarity to the protein profile of the WM extract produced from the standard liquid culture preparation of *C. puteana* mycelia (tracks 2, 17). Similarly the profiles of the organism grown on cellulose (tracks 4, 6, 8, 10, 12,

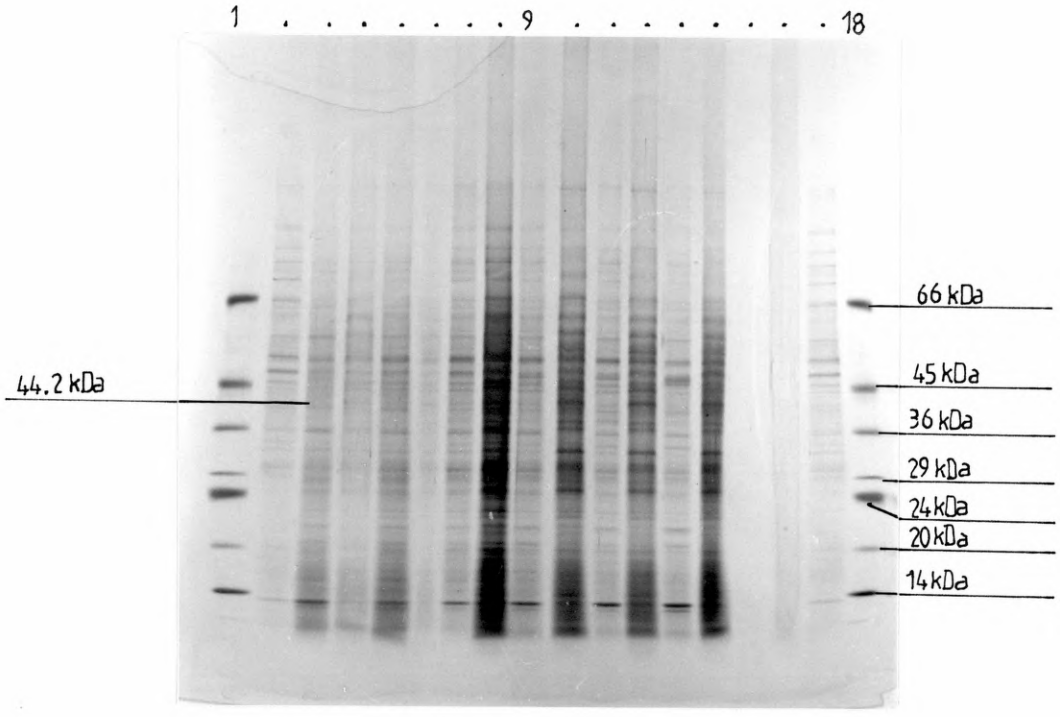


FIGURE 4.7. SDS-PAGE analysis of protein profiles of 2, 4, 6, 8, 10 and 12 day harvests of WM extracts of agar cultured *C. puteana* compared to that of cellulose cultured *C. puteana*. Tracks represent: 1, 18 - MWM (Figure 4.3.); 2, 17 - *C. puteana* FPRL 11E; 3, 5, 7, 9, 11, 13 - 2-12 day harvests of *C. puteana* grown on agar medium; 4, 6, 8, 10, 12, 14 - 2-12 day harvests of *C. puteana* grown on cellulose medium; 15 - agar medium control; 16 - cellulose medium control.

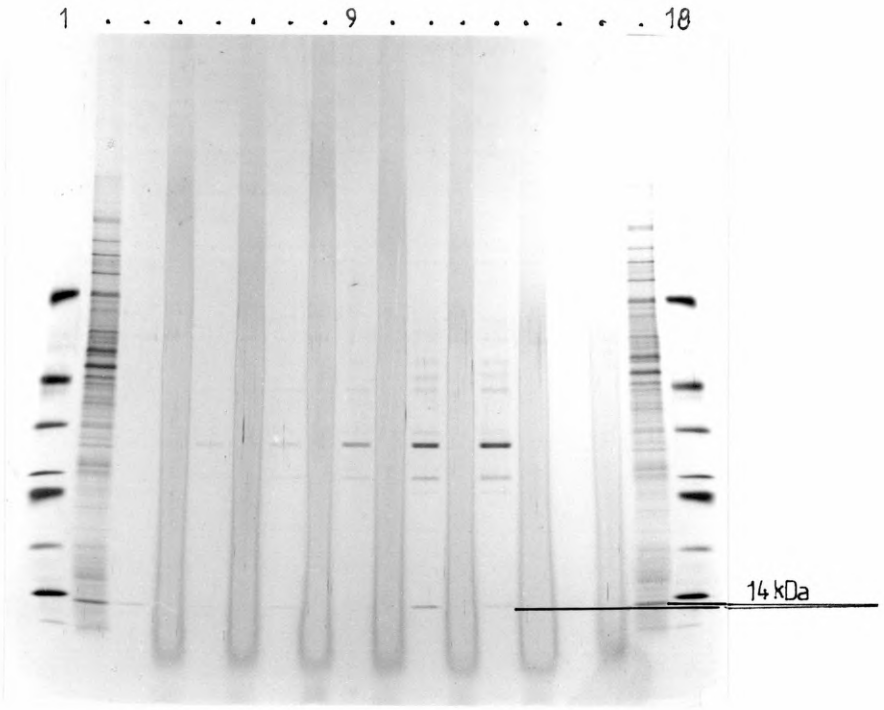


FIGURE 4.8. SDS-PAGE analysis of protein profiles of 2, 4, 6, 8, 10 and 12 day harvests of EP extracts of agar, cellulose and liquid cultured *C. puteana* compared to that of cellulose cultured *C. puteana*. Tracks represent: 1, 18 - MWM (Figure 4.3.); 2, 17 - WM extract of *C. puteana* FPRL 11E; 3, 5, 7, 9, 11, 13 - 2-12 day EP extracts of *C. puteana* grown on agar medium; 4, 6, 8, 10, 12, 14 - 2-12 day EP extracts of *C. puteana* grown on cellulose medium; 15 - agar medium control; 16 - cellulose medium control.

14) were substantially the same as the profiles produced when grown on liquid or agar culture. In both cases there were a number of minor changes against a consistent background pattern. That the proteins evident were not artifacts of media was clearly shown as extracts of the culture media contained little or no protein (tracks 15, 16). Culture age had a limited effect on the protein profile of *C. puteana* when grown on agar medium, for example, a protein of approximate molecular weight 44,200 Da which was present at 2 days of growth, became more evident from 4 days growth to 12 days growth. The profile exhibited by *C. puteana* when grown on cellulose medium only showed minor changes during growth, no enhancement of the 44,200 Da protein was seen.

Figure 4.8., indicated that it was not possible to detect EPs by SDS-PAGE when *C. puteana* was grown on cellulose (tracks 4, 6, 8, 10, 12, 14). EPs could however be detected when the organism was grown on agar medium (tracks 3, 5, 7, 9, 11, 13). One protein was evident at only 2 days of growth, 8 proteins being evident at 12 days of growth. The protein profile exhibited by EP extraction was different from that of the WM standard preparation (tracks 2, 17) although some of the proteins were similar, for example the protein of approximate molecular weight of 14,000 Da.

4.4.3. MOLECULAR ANALYSIS OF SAWDUST CULTURED *C. PUTEANA*

The effect of sawdust grown *C. puteana* on protein profiles was determined by the preparation of WM and EP extracts after 2, 4, 6, 8 and 10 days of growth. The results shown in Figure 4.9., indicate that as the organism was cultured

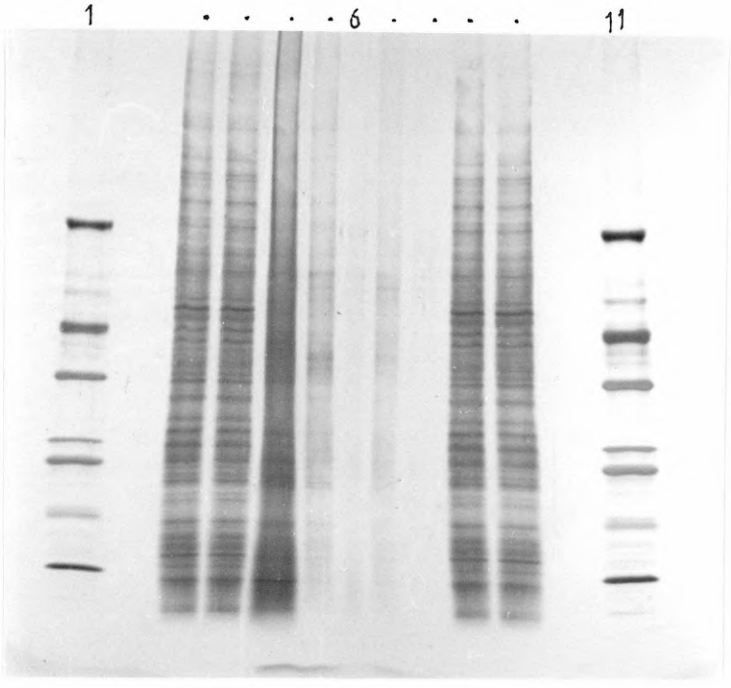


FIGURE 4.9. SDS-PAGE analysis of protein profiles of WM extracts of liquid cultured *C. puteana* compared to sawdust cultured *C. puteana* harvested at 2, 4, 6, 8 and 10 days of growth. Tracks represent: 1, 11 - MWM (Figure 4.3.); 2, 3, 9, 10 - *C. puteana* FPRL 11E; 4, 5, 6, 7, 8 - *C. puteana* grown on sawdust medium and harvested at 2-10 days respectively.

on sawdust medium the protein bands within the profile of WM extracts gradually became less intense. The protein profile which was apparent at 2 and 4 days of growth (tracks 4, 5) however, was similar to the profile of the liquid preparation of *C. puteana* (tracks 2, 3, 9 and 10). No EPs were detected in extracts of sawdust grown *C. puteana*.

4.4.4. IMMUNOLOGICAL ANALYSIS

The molecular analysis of *C. puteana* indicated that despite the age or region of mycelia or the type of sustrate, the profile of the organism remained similar to the profile of the standard preparation of FPRL 11E (Figure 4.7.), thus facilitating identification and detection of *C. puteana*. An investigation of the antigenic nature of *C. puteana* was carried out under identical conditions to that described in sections 4.4.2. and 4.4.3. Specifically, the consistency of antigen expression over time was studied in conjunction with the analysis of antigen expression when *C. puteana* was cultured on different substrates. In addition, the immunogenicity of extracts was examined since highly immunogenic extracts might be useful in the production of further reagents.

Figure 4.10., which shows results for analysis of WM extracts produced from *C. puteana* grown on liquid, agar, cellulose and wood substrates and probed with antiserum 88/1, indicates that the antigenic profile of extracts of agar (tracks 5, 7, 9, 11, 13, 15) and cellulose (tracks 4, 6, 8, 10, 12, 14) grown *C. puteana*, is similar to that of the standard preparation (track 2). The antigenic nature of *C. puteana* was also unaffected by culture age, when grown

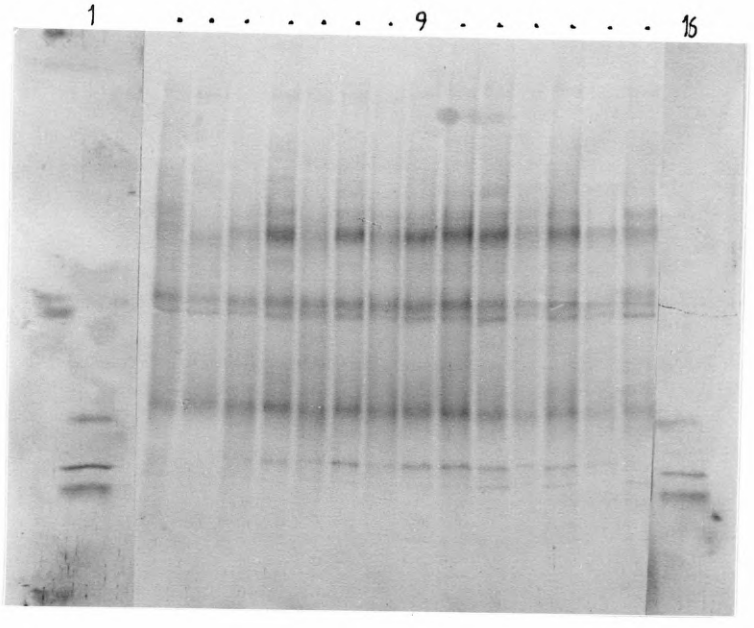


FIGURE 4.10. Western blotting analysis of antiserum 88/1 probed antigen profiles of WM extracts of agar cultured *C. puteana* compared to that of cellulose cultured *C. puteana*. Tracks represent: 1, 16 - MWM (Figure 4.3.); 2 - *C. puteana* FPRL 11E; 3 - 34.12% weight loss wood block; 4, 6, 8, 10, 12, 14 - 12, 10, 8, 6, 4 and 2 day harvests of *C. puteana* grown on cellulose medium; 5, 7, 9, 11, 13, 15 - 12, 10, 8, 6, 4 and 2 day harvests of *C. puteana* grown on agar medium.

on all substrates including wood (track 3, weight loss 34.12%).

The antigen profiles of WM extracts of *C. puteana* grown on sawdust medium are shown in Figure 4.11. Due to the lack of material it was difficult to produce high quality results for the analysis, however, Figure 4.11.(i) indicates that the antigen profiles were similar to that of the standard preparation (track 6). The profile became less intense as the organism was cultured on sawdust agar from 2-10 days (tracks 5, 4, 3, 2, 1, respectively).

The growth of *C. puteana* on sawdust medium suggests the presence of mainly growing tip mycelia (Figure 4.6.(ii)). To determine if this mycelia contained different antigens to the standard extract of *C. puteana*, antigens which may be unique to the growing tip region, an analysis was undertaken using the growing tip antiserum 90/2 (Figure 4.11.(ii)). Only one antigen was detected in each sample analysed (cf. the standard extract, track 7) and this antigen was less obvious with increased growth on sawdust medium. One of the major WM antigens was found to have the same molecular weight as the antigen evident in Figure 4.11.(ii) (WM Ag 2; 98,900-92,300 Da).

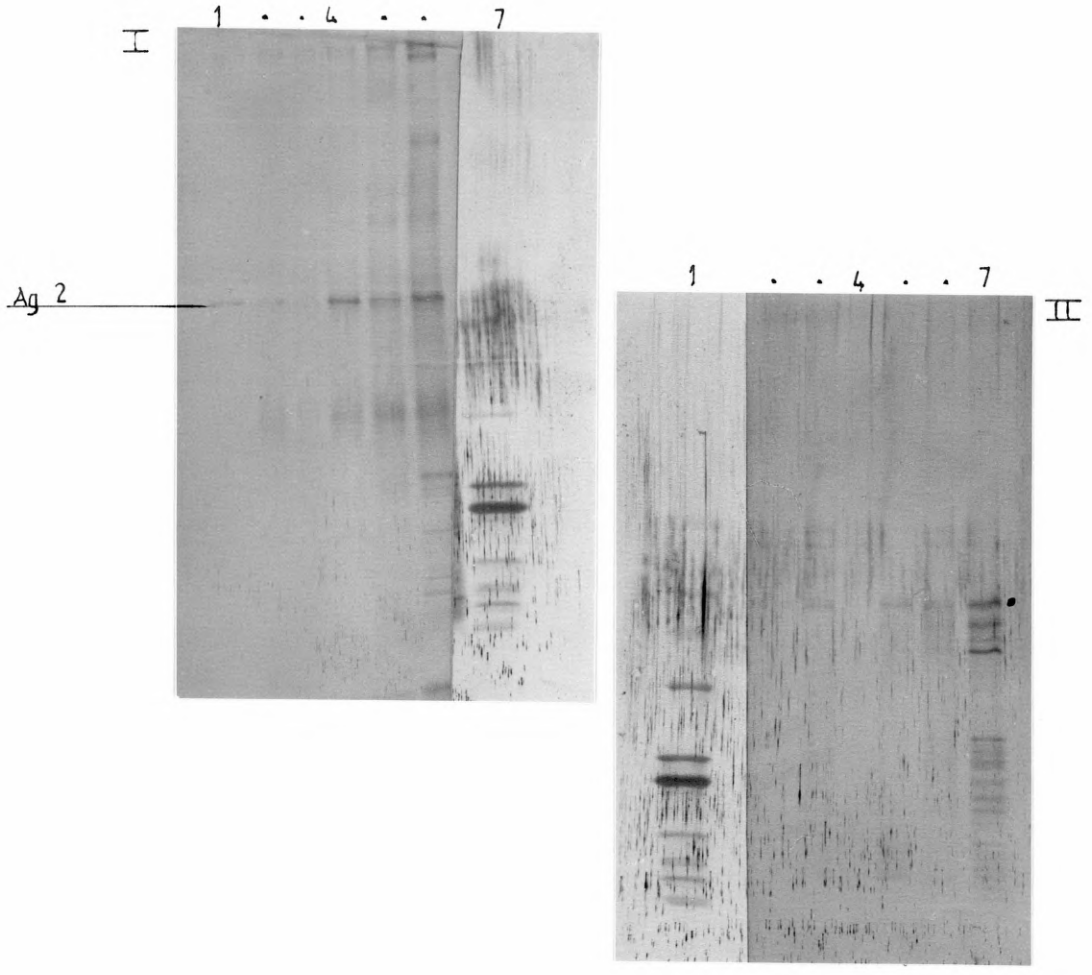


FIGURE 4.11. Western blotting analysis of the antigen profile of WM extracts of *C. puteana* cultured on sawdust medium compared to that produced on liquid medium. PART I - antiserum 88/1; Tracks represent: 7 - MWM (Figure 4.3.); 1, 2, 3, 4, 5 - *C. puteana* grown on sawdust medium and harvested at 10, 8, 6, 4 and 2 days; 6 - *C. puteana* FPRL 11E. PART II - antiserum 90/2; Tracks represent: 1 - MWM (Figure 4.3.); 2, 3, 4, 5, 6 - *C. puteana* grown on sawdust medium and harvested at 10, 8, 6, 4 and 2 days; 7 - *C. puteana* FPRL 11E.

4.5. ANALYSIS OF THE PROFILES PRODUCED FROM EXTRACTS OF THE DISTINCT REGIONS OF *C. PUTEANA* MYCELIA: GT, INTERMEDIATE AND AGED

4.5.1. MOLECULAR ANALYSIS

Studies by Vigrow *et al.*, (1991), indicated that the GT protein profile of *S. lacrymans* was different to that of the standard and to that of aged mycelial preparations of the organism. To investigate this for *C. puteana* 3 distinct regions of the mycelium which are evident in an agar culture of the organism (Figure 4.5.(ii)) were analysed by SDS-PAGE. An analysis of EP extracts of these mycelial preparations was also undertaken.

Figure 4.12. shows the protein profiles obtained for WM and EP extracts of the various regions of the *C. puteana* mycelium. Tracks 2, 15 and 3, 14 show the standard profiles for WM and EP extracts of *C. puteana* respectively (see Figures 3.2. and 3.10.). The protein profiles for the WM extracts of the intermediate and GT regions of the fungal mycelium (tracks 12 and 13) were substantially similar to the standard WM profile (track 2, 15) though some differences in the intensity of certain proteins were noted. In addition the aged mycelial profile (track 11) has bands in common with the intermediate and GT profiles, but also shows a resemblance to the standard EP profile (tracks 3, 14). A greater variation in protein profiles was evident for EP extracts of the different regions of the *C. puteana* mycelium compared to the WM extracts of these regions. The aged mycelial profile (track 6) had similarities to the standard EP extract profile (tracks 3 and 14), and the GT mycelial profile (track 8) had similarities to the WM

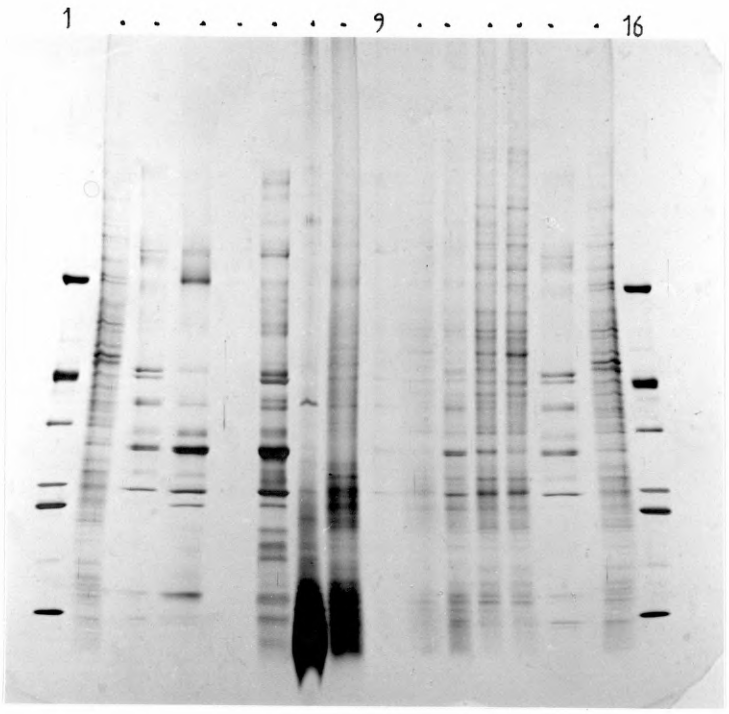


FIGURE 4.12. SDS-PAGE analysis of protein profiles of WM and EP extracts of GT, intermediate and aged mycelial regions of *C. puteana*. Tracks represent: 1, 16 - MWM (Figure 4.3.); 2, 15 - WM standard; 3, 14 - EP standard; 4 - aged EP mycelia (EAV); 5 - GT EP mycelia (EAV); 6 - aged EP mycelia; 7 - intermediate EP mycelia; 8 - GT EP mycelia; 9 - aged WM mycelia (EAV); 10 - GT WM mycelia (EAV); 11 - aged WM mycelia; 12 - intermediate WM mycelia; 13 - GT WM mycelia.

profiles of *C. puteana* (tracks 9-13) although many low molecular weight proteins were evident in the GT profile. The intermediate protein profile (track 7) exhibited mainly low molecular weight proteins which may be similar to those present in the EP and GT extracts and all WM extracts. Using the method of Vigrow (2.3.3.) it was not possible to produce profiles of sufficient intensity (tracks 4, 5, 9, 10), however such proteins as can be detected appeared identical to those found using the methodology described in this section.

4.5.2. IMMUNOLOGICAL ANALYSIS

Section 4.5.1., indicated that the protein profiles of WM extracts from the GT, intermediate and aged regions of the *C. puteana* mycelia were similar, but variation existed for EP extracts of these regions. The antigens present in these regions were examined to establish their similarities and differences. In order to maximise the chance of finding variability in antigen profiles, 3 polyclonal antisera (detailed below) were utilised for the analysis of WM and EP extracts of the different mycelial regions. The antisera included i) 88/1, the antiserum raised against WM extracts, ii) 88/8, raised against EP extracts and produces exoantigen profiles as detailed in chapter 3, and iii) 90/2, raised against GT extracts which may provide information on growth phase antigens. A comparison of these results is shown in Table 4.1.

ANTISERA	MYCELIAL REGION	EXTRACT OF MYCELIAL REGION	
		WM	EP
88/1 WM	GT	+	+
	I	+	+
	A	+	x
88/8 EP	GT	-	-
	I	-	-
	A	-	x
90/2	GT	o	o
	I	o	-
	A	o	x

KEY

GT - Growing tip extract

I - Intermediate extract

A - Aged extract

\+ - Similar to standard WM profile i.e. WM/88/1

x - Similar to standard EP profile i.e. EP/88/8

o - Similar to standard GT profile i.e. GT/90/2

\- - Not possible to analyse.

TABLE 4.1.

SUMMARY OF ANALYSIS OF ANTISERA 88/1, 88/8 AND 90/2 AND VARIOUS EXTRACTS OF *C. PUTEANA*

4.5.2.1. WM ANTISERUM (88/1)

The 3 different regions of the fungal mycelium were analysed using antiserum 88/1 and results are shown in Figure 4.13. All WM extracts (tracks 8, 9, 10) appeared similar in their antigen profiles to each other and to the standard WM profile (tracks 2, 6 and Figure 4.3.). The profiles of the GT (track 3) and intermediate (track 4) EP extracts also shared similarities with the standard WM profile, while the profile of the aged EP extract shared a greater resemblance to the standard EP profile (Figure 4.3.) The blot further illustrates difficulties encountered in the analysis of EP extracts, notably in tracks 3 and 4.

4.5.2.2. EP ANTISERUM (88/8)

Few bands were detected in most samples analysed (Figure 4.14.)., however one of the antigens present in the standard EA profile (33,100 Da; tracks 7, 11) was also present in the profiles of all extracts, except for the profile of the intermediate EP extract (track 9). In addition the antigen with the approximate molecular weight of 45,700 Da present in the standard EA profile (tracks 7, 11) was also present in the aged EP extract profile (track 10).

4.5.2.3. GT ANTISERUM (90/2)

Figure 4.15. shows the analysis of extracts of *C. puteana* using antiserum 90/2. In comparison to the EA blot (Figure 4.14.) very strong profiles were found in this analysis. Variation in the antigen profile obtained for WM extracts was limited to a reduction in the number of antigens

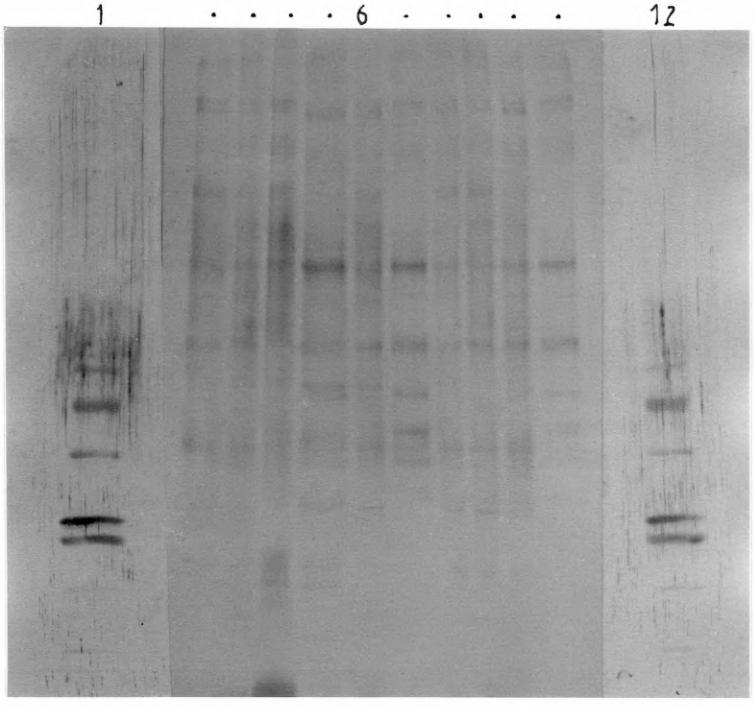


FIGURE 4.13. Western blotting analysis of different regions of *C. puteana* mycelia using antiserum 88/1. Tracks represent: 1, 12 - MWM (Figure 4.3.); 2, 6 - WM standard; 3 - GT EP extract; 4 - intermediate EP extract; 5 - aged EP extract; 7, 11 - EP standard; 8 - GT WM extract; 9 - intermediate WM extract; 10 - aged WM extract.

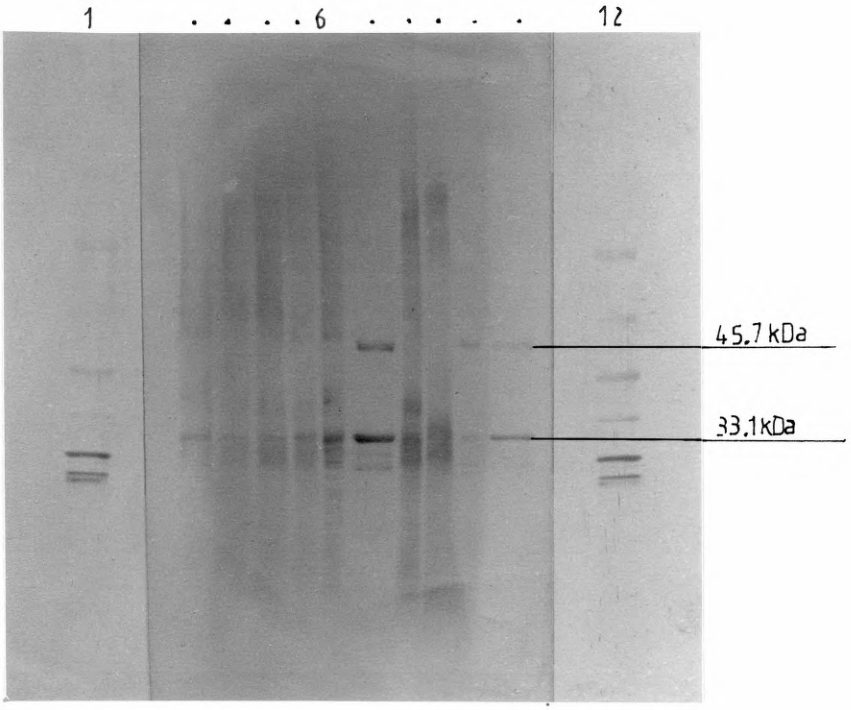


FIGURE 4.14. Western blotting analysis of different regions of *C. puteana* mycelia using antiserum 88/8. Tracks represent: 1, 12 - MWM (Figure 4.3); 2, 6 - WM standard; 3 - GT WM extract; 4 - intermediate WM extract; 5 - aged WM extract; 7, 11 - EP standard; 8 - GT EP extract; 9 - intermediate EP extract; 10 - aged EP extract.

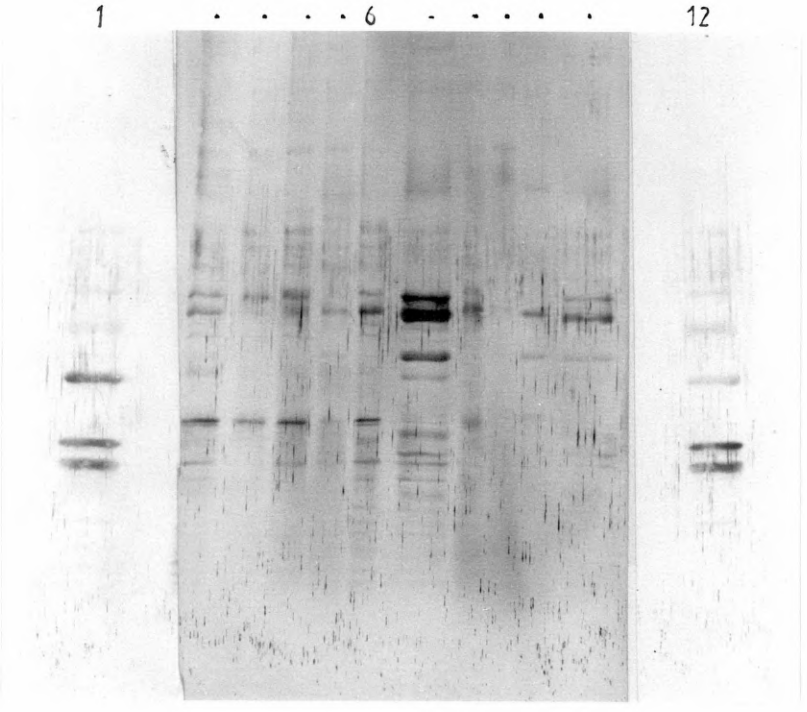


FIGURE 4.15. Western blotting analysis of different regions of *C. puteana* mycelia using antiserum 90/2. Tracks represent: 1, 12 - MWM (Figure 4.3.); 2, 6 - WM standard; 3 - GT WM extract; 4 - intermediate WM extract; 5 - aged WM extract; 7, 11 - EP standard; 8 - GT EP extract; 9 - intermediate EP extract; 10 - aged EP extract.

detected in the aged mycelial sample (track 5). Similar antigens to those present in the profile of the WM standard were evident for the profile of the GT EP extract (track 8). No major antigens were detected in the intermediate EP extract profile (track 9). The profile of antigens for the aged mycelial EP extract (track 10) was more similar to the antigens of the standard EP extract (track 11) than the antigen profiles of the GT EP extract (track 8) or the profiles of the WM extracts (tracks 2-6).

4.6. DISCUSSION

It was established in the 1860's by Jules Raulin, a student of Pasteur, that changes in nutrition had a direct affect on the growth of fungal organisms. Raulin set out to examine the mineral nutrition of *Aspergillus niger* and in doing so established a protocol for controlling the physical as well as the chemical environment of the media. The outcome of the analysis was the production of defined media for the reproducible growth of particular organisms. The resultant growth was either a reflection of organism growth in nature, or a representation of optimum growth (Garraway and Evans, 1984).

Individual fungi are quite selective in their nutritional requirements and normally metabolise only a restricted set of substrates. In addition if the nutrient supply is depleted or is in a form which is inaccessible, the morphology of growth is likely to be affected. For example, the cellular slime mould *Dictyostelium* grows as an amoeboid cell, however when the food supply is exhausted, the amoebae aggregate to form a snail-like structure (Brock *et al.*, 1979). Similarly, depending on the environment other

fungi can switch from a mycelial to a non-mycelial phase, for example, *Mucor rouxii* changes to a yeast-like form under elevated carbon dioxide levels (Garraway and Evans, 1984). *C. albicans*, also under the appropriate conditions, has the ability to switch from a mycelial to a non-mycelial phase, the latter resulting in the pathogenic growth of the organism and human disease (Odds, 1988).

Wood decay basidiomycetes also have the ability to alter their morphology when subject to adverse conditions, growing as nutrient searching mycelia under normal conditions in wood, producing hyphal strands when nutrients are low and finally fruiting bodies when the nutrient source is depleted, or as a direct result of adverse environmental pressures (Ginns, 1982).

In addition to gross morphological changes, more subtle changes within the mycelia of some basidiomycetes are also evident. For example, in agar cultures, *S. lacrymans* exhibits point growth under conditions which may be unfavourable to the organism although no conclusive reason for this type of growth is available (Jennings, 1991; Vigrow, 1992). *L. lepideus* grows randomly on liquid culture, but on agar it is seen as an even advancing mycelial front growing from an individual inoculum (Bruce, personal communication). Such changes for *C. puteana* growing on liquid (standard), agar, cellulose and sawdust media were therefore investigated. Cellulose and sawdust media were examined since these more closely reflect the growth of *C. puteana* in nature.

Particular alterations in the growth of *C. puteana* were observed. Random growth was evident on liquid culture and consistent radial growth was evident on agar culture, similar to that observed for *L. lepideus* (Bruce, personal communication). Both media types however provided healthy and optimum growth of *C. puteana* which was consistent with that described by Ginns (1982). However when *C. puteana* was grown on the media, which were most likely to reflect the nutritional sources available in nature, its growth was severely restricted; the mycelia produced exhibiting a thinning of hyphae, appression and lack of aerial growth. The only floccose parts of the growing colonies were the hyphal tips which appeared to be the only healthy part of the colony. Such variation in growth might be a reflection on the availability of the nutrients within the media and the ability of *C. puteana* to access these nutrients.

A source of nitrogen is indispensable for fungal growth and development. It is essential for the synthesis of a variety of critically important cellular constituents including amino acids, proteins and vitamins (Garraway and Evans, 1984). The availability of nitrogen in the cellulose and sawdust medium was very low, but sufficient to allow some growth of *C. puteana*. Growth was less in the media containing least nitrogen (cellulose). The concentration of nitrogen in these media may not be high enough to allow the substantial utilisation of the other media components, thus restricting growth.

These changes however clearly do not rely, in *C. puteana* at least, on specific alterations of WM protein and antigens of this organism since no such alterations were observed. When other wood decay fungi, for example, *S. lacrymans*, *L.*

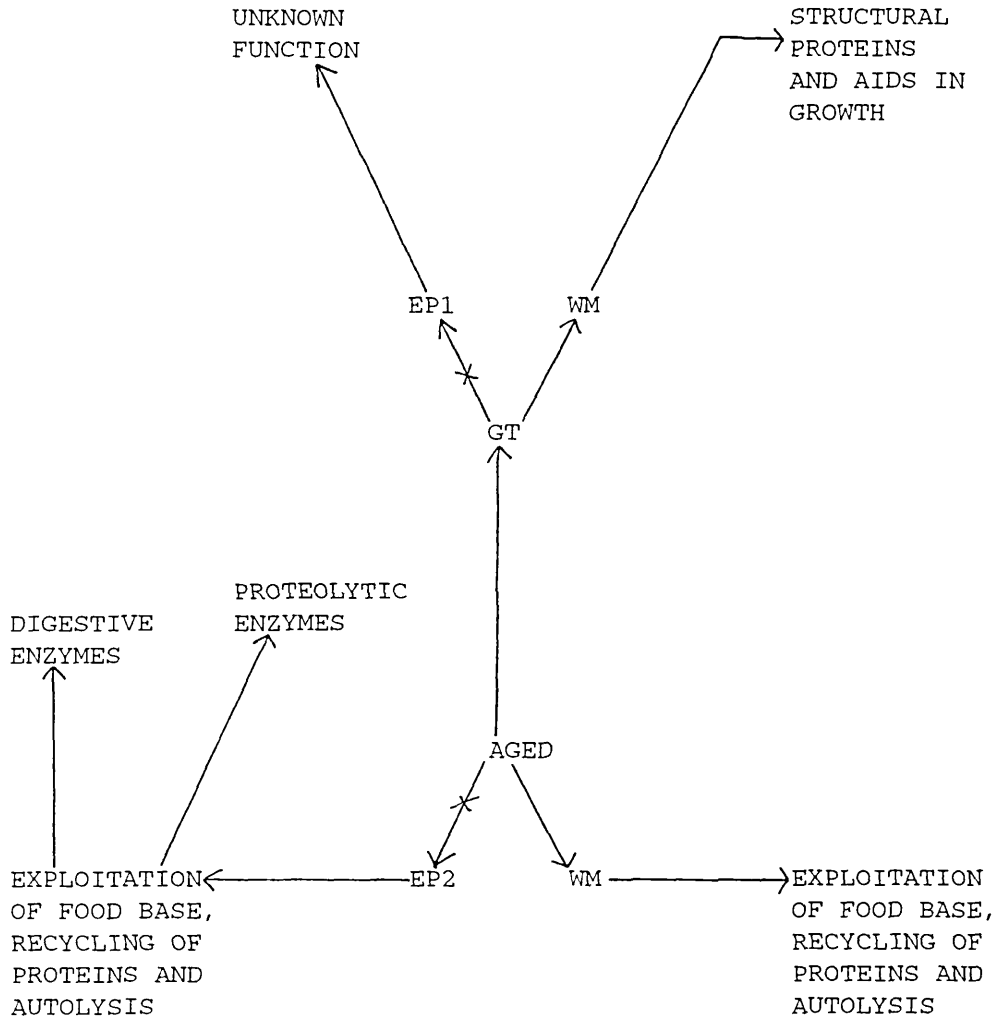
lepideus and *C. versicolor*, are cultured on different media containing sufficient nutrients a high degree of similarity within protein and antigen profiles is also observed (Palfreyman *et al.*, 1988; Vigrow *et al.*, 1989; Glancy, 1990). However when fungi are cultured on media which do not contain sufficient nutrients changes in the mycelia similar to those observed for *C. puteana* are evident. For example, *S. lacrymans* cultured on media with varying nitrogen levels exhibited sparse, appressed growth which was directly attributed to the nitrogen content of the media. The endo- proteins and antigens of these mycelia were however, unlike those of *C. puteana*, affected by the nitrogen levels since the intensity of specific bands either increased or decreased as the total nitrogen content of the medium was reduced (Vigrow, 1992).

Exoprotein profiles for *C. puteana* were described in chapter 3, for the organism grown on agar cultures. However no EP or EA profiles were detected from *C. puteana* extracts after culture of the organism on sawdust or cellulose media. The lack of evidence of EP/EA profiles may be due to the fact that the food base available was not in a suitable form, or lacked nitrogen for optimal growth. In addition, EP/EAs may be more susceptible to induction or repression than whole cell components in reaction to nutrient availability (Venables and Watkinson, 1989). Alternatively, the lack of detection of EP/EAs may be directly due to a concentration effect, the greater the biomass of mycelia, the more abundant the EP/EAs. In either case, evidence of floccose mycelia only at the growing edge of the colony suggests that most metabolic energy was directed towards exploration of the food base. The production of proteins not essential for this exploration could therefore be

inhibited to conserve energy (Venables and Watkinson, 1989). Since some degradation of the media components did occur it is possible that EPs are not essential for degradation, but may facilitate it, thus increasing the growth rate of the organism (cf. the growth rate of agar cultured *C. puteana* to cellulose cultures of this organism).

EP/EAs were produced on agar medium, the full profile becoming evident at about day 5 of growth suggesting that satisfactory nutrients were available in the media to stimulate their production. Hence the lack of EP/EAs as discussed above is unlikely to be due to a concentration effect, but to the absence of suitable nutrients. However these results contrast with the evidence of the immediate presence of the majority of internal proteins of the WM extract at an early stage of growth even under such adverse conditions. This suggests that the WM proteins are fundamental proteins necessary for the basic structure of the hyphae, whilst the EPs are required for functions not directly related to the initial formation of hyphae. WM proteins and EPs will now be discussed with reference to Figure 4.16.

When cultured on agar media, *C. puteana* mycelia exhibited 3 regions showing different morphology. This is analogous to *S. lacrymans* in which at least 2 morphological regions of growth have been recognised; the growing tip and the aged mycelial regions (Vigrow, personal communication; Hornung and Jennings, 1981). The 3 regions of *C. puteana* represent; the growing hyphal tip, which may be necessary for the exploration of food bases (Watkinson, 1975); the intermediate mycelia, the function of which may be for the



KEY

EP1 - EPs produced from GT mycelia
EP2 - EPs produced from aged mycelia
WM - Whole mycelial proteins
X - Pathway reduced when *C. puteana* is grown on cellulose or sawdust

FIGURE 4.16. Summary of the production and possible functions of EPs of *C. puteana*.

main exploitation phase of growth, involving the production of enzymes for the degradation of the substrates and the recycling of components from the aged mycelia to the growing tip, for further extension of the colony; and the aged mycelia, which may be necessary for the degradation of proteins for the recycling of nutrients to the GT or for the production of strand material at low nutrient availability (Watkinson, 1975).

The analysis of WM extracts of the protein and antigen profiles of the 3 morphological regions of *C. puteana* was necessary to further establish simple profiles for identification and detection purposes. The results indicated that the GT and intermediate WM extracts have similar protein and antigen profiles to that of the standard WM extract. The WM aged mycelial extract of *C. puteana* contained fewer protein species than the other WM extracts and in addition was more similar to the protein profile of the standard EP extract than that of the standard WM extract. The difference between the GT and aged WM profiles is in agreement with results reported for *S. lacrymans*. Vigrow *et al.*, (1990), suggested that the GT region of *S. lacrymans* might contain proteins specifically associated with the exploration phase of growth of the organism. However the similarity between the GT and intermediate profiles of *C. puteana* suggests that the difference between the GT and the aged profiles may also reflect proteins produced during recycling and senescence. The reduction in proteins in the aged mycelia and the fact that they are mainly of low molecular weight may therefore be due to proteins being degraded for recycling for use by the growing hyphal tips.

The protein and antigen profiles of the WM aged mycelia of *C. puteana* share many similarities with the profiles of the EP/EA standards. This suggests that the WM proteins detected in the aged mycelia, may become EP/EAs when they are released from the hyphae. The definition of EAs by Kaufman and Standard (1987), describes the release of EAs by fungal organisms early in their growth. This suggests that EAs may be produced from the growing tips of the organism. However the results described suggest that EAs are released from aged mycelia. The abundance of EP/EA in aged mycelia of *C. puteana* may have some correlation with the discovery by Venables and Watkinson (1989) and Kalisz *et al.*, (1989) of the presence of proteases produced from the aged mycelia of *Gloeophylum trabeum*, *L. lepideus*, *S. lacrymans* and *Phlebia gigantae* rather than in the growing tips of the colony. Proteases produced from the aged mycelium are required for the retrieval of the fungal protein from senescent mycelia; perhaps this is the function of some of the exoproteins of *C. puteana* aged mycelia. The profile of the intermediate EP and GT EP extracts of *C. puteana* are substantially different from the aged mycelial EP profile. If the function of EPs is related to some kind of recycling of protein components, then perhaps these profiles (Figure 4.12.) represent proteases responsible for the recycling of proteins and partially degraded proteins, which are being recycled to the GT for the extension of the colony. This is reflected by the presence of low molecular weight proteins in the aged EP and intermediate EP extracts compared to the increased presence of high molecular weight proteins in the GT EP profile.

The exoprotein profile which is obtained for *C. puteana* is similar to an SDS-PAGE total protein profile for an extract of filtrate from batch cultured *C. puteana* (Schmidhalter and Canevascini, 1990). The proteins described by Schmidhalter and Canevascini, have the approximate molecular weights of 84,000 Da, 48,000 Da and 36,000 Da while those described in this chapter have weights of 76,000 Da, 46,000 Da, 38,000 Da, 33,000 Da, 32,000 Da and 21,000 Da. Schmidhalter and Canevascini, have identified some of these proteins as cellobiohydrolases which are glycoproteins with degradative enzymic activity on amorphous cellulose. It is possible therefore that the EPs identified for *C. puteana* in this chapter, consist of glycoproteins which are involved in some stage of the decay process, and proteases involved in recycling proteins. Three lines of evidence which may substantiate the hypothesis described for *C. puteana* include; the detection of glycoproteins in surface washings of the fungal organism *H. lanuginosa* (MacDonald *et al.*, 1989); the detection of exocellular proteases of *S. lacrymans* by Venables and Watkinson (1989) in aged mycelia of this organism and the fact that Griffin in 1981 indicated that exocellular proteins tend to be proteases which are exo or endohydrolases.

The initial studies in this chapter indicated that the antigens detected by antiserum raised against the whole cell mycelial extract were not susceptible to change due to substrate differences but the production of exoantigens was affected. In addition, a greater variation in the exoantigens was evident when different parts of the mycelia of *C. puteana* were analysed using antisera raised against WM extracts and EP extracts. With the knowledge that

variation of exoproteins exist it was necessary to investigate these antigens further in comparison to WM and GT antigens, specifically by analysing WM, GT antigens and EAs using antisera raised against all 3 immunogens.

Antiserum 88/1 raised against WM antigens was used as a reference in this analysis since studies of other wood decay fungi have always used WM extracts. That the GT WM antigen profile of *C. puteana* was similar to the standard WM profile using this antiserum was evident and contrasts with results described for *S. lacrymans* in which the GT WM extract exhibited antigens which were different from the standard WM extract and which resembled more closely the profile produced for pine and lime sapwood grown material (Vigrow *et al.*, 1990) (see chapter 5 for *C. puteana* infected wood). The fact that there is similarity of the GT WM profile of *C. puteana* to the standard WM profile, indicates that specific growth phase antigens cannot be detected using antiserum 88/1 (see also chapter 5).

The analysis using antiserum 88/1 also indicated that the exoantigens of *C. puteana* exhibited a similar profile to that of the GT WM and the standard WM profiles, but also exhibited unique antigenic bands. These EAs must be present in low concentrations in the WM extract preparation to allow a reaction to occur with the WM antiserum. This suggests that EAs are highly immunogenic.

The exoprotein antsera (88/8) detected mainly low molecular weight antigens. This is consistent with the detection of low molecular weight glycoproteins in surface washings of fungal cultures of *H. lanuginosa* (MacDonald *et al.*, 1989). Low molecular weight antigens were also found in the

antigen profiles of GT WM and standard WM extracts when reacted with antiserum 88/8, some of which were similar to the exoantigens exhibited in the standard EA profile. This suggests that antiserum 88/8 may be reacting with internal cellular components which may be exoantigens within the WM extract which have not yet been released.

The GT antisera was prepared by the immunization of rats with unwashed GT material and therefore the antisera produced contains antibodies to internal proteins as well as exoproteins. Since the antigen profiles produced for the reaction of all extracts with the GT antiserum (90/2) were similar to each other, and also to the standard EA profile with antiserum 88/8, the exoantigens must be more immunogenic than the GT or the WM extract antigens. If EAs are more immunogenic and antigenic than the GT and WM antigens, this renders them ideal as immunological detectors and receptors, especially in the production of monoclonal antibodies. That they are more immunogenic and antigenic may also have a bearing on the high cross-reactivity displayed by their antiserum, e.g. 88/8, and antisera to *P. placenta*, *L. edodes*, *T. palustris* and *C. versicolor* (Kim *et al.*, 1991).

4.6.1. CONCLUSIONS

Morphological changes in mycelia apparent for *C. puteana* when subjected to different substrates, clearly had no direct consequence on specific protein and antigens within the hyphae of the organism, whilst the surface exoproteins and antigens were affected. The internal mycelial proteins of the GT and intermediate regions are different from those of the aged mycelial region and appear to be required for

the growth and structure of the organism. The internal mycelial proteins of the aged region are similar to the standard EP/EA profiles, which suggests that these exoproteins are more abundant in aged mycelia of *C. puteana*. In addition, the EP extract is highly immunogenic, rendering these extracts more useful than the WM extracts for the production of monoclonal antibodies to specifically detect *C. puteana* in wood.

CHAPTER FIVE

WOOD DECAY STUDIES

5.1. INTRODUCTION

A molecular examination of *C. puteana* infected wood is a necessary prerequisite for the development of molecular identification systems for the recognition of *C. puteana*, since wood is the natural substrate of the organism. Similarly a comparative analysis of the antigenic characteristics of the organism in wood to that in liquid culture, is essential for the immunological detection of *C. puteana* in the field. The main objective of the research described in this chapter is therefore to study the possibility of identifying and detecting *C. puteana* in wood, by the application of molecular and immunological techniques discussed in previous chapters.

5.2. THE ANALYSIS OF THE PROTEIN/ANTIGENS OF *C. PUTEANA* IN WOOD

Pine (*P. sylvestris*) sapwood blocks ($1cm^3$) were decayed by *C. puteana* for periods of up to 6 weeks (2.15.), producing blocks with percentage weight losses in the range 0-35%. Details of the weight losses of individual blocks can be found in Table 5.1. Whilst in general, weight losses correlated with length of incubation, some anomalous results were found. The anomalous results may be due to water-logging of blocks which were subject to excessive water uptake from the agar, consequently delaying the invasion of the blocks by *C. puteana* mycelia (e.g. blocks 25, 26, 29). Overall the mean weight loss values obtained, if the anomalous values are disregarded, indicate that as the exposure time of the wood blocks to *C. puteana* increased from 0-6 weeks, the average percentage weight losses of the blocks increased, indicating continuous

PERIOD OF DECAY	WOOD BLOCK NO.	% WT. LOSS	
0 WKS (uninfected)	66	0.57	
	37	1.90	
	89	0.57	
	85	0.08	
	31	0.29	
	76	0.38	AV - 0.63
2 WKS	41	0.19	
	45	0.92	
	47	0.32	
	46	0.76	
	48	2.72	
	42	1.01	AV - 0.10
4 WKS	25	2.84*	
	26	3.04*	
	27	26.48	
	28	16.74	
	29	7.45*	
	32	26.27	AV - 23.16
6 WKS	33	29.02	
	35	2.71*	
	36	2.99*	
	38	17.82	
	39	1.63*	
	57	34.12	AV - 26.95

KEY
WKS - Weeks
WT - Weight
AV - Average % wt. losses excluding anomalous results
NO - Number
* - Anomalous % wt. losses

TABLE 5.1.

PERCENTAGE WEIGHT LOSSES OF BLOCKS DECAYED BY *C. PUTEANA* FPRL 11E FOR A MAXIMUM OF 6 WEEKS

degradation. Table 5.2. shows the individual wood blocks, utilised in subsequent analyses and their associated percentage weight losses. Also included in Table 5.2., are a further 2 blocks with weight losses greater than 40% which were selected for analysis from the experiment described in 5.3. Selection of these blocks allowed an investigation of the molecular and immunological nature of *C. puteana* during advanced decay (high percentage weight losses).

5.2.1. MOLECULAR ANALYSIS OF *C. PUTEANA* INFECTED WOOD BLOCKS

The molecular and immunological analysis of wood blocks with a range of percentage weight losses (Table 5.2.) are shown in Figures 5.1. and 5.2. respectively. The standard protein profile of FPRL 11E (tracks 2, 7, 13) was compared to the protein profiles for extracts of a range of FPRL 11E decayed wood blocks (Figure 5.1., tracks 5-6; 8-11). The profiles of these extracts are substantially similar to each other and to the profile of FPRL 11E (tracks 2, 7, 13). These profiles are evident once losses of 16.74% (track 6) or greater are recorded and indicate that *C. puteana* can be identified at these weight losses. However, at 16.74% weight loss the percentage similarity index value is only 59% (Table 5.3.), which is below the level required to make a positive identification as *C. puteana* (chapter 3). At a weight loss of 26.48% or greater (track 8), similarity index values of >60% are found, consistent with the specific identification of *C. puteana*. With the exception of a protein with the approximate molecular weight of 14,200 Da which is present in the later stages of decay of *C. puteana* infected wood blocks (tracks 10, 11),

WOOD BLOCK NO.	% WEIGHT LOSS
85	0.08*
45	0.92
28	16.74
27	26.48
57	34.12
60	49.15
86	54.73

KEY
* - Uninfected wood block represented as 0% weight loss or uninfected (U) in subsequent analyses.
NO - Number

TABLE 5.2.

PERCENTAGE WEIGHT LOSS BLOCKS USED IN SUBSEQUENT ANALYSES

TEST	0.0	0.92	16.74	26.48	34.12	40.0	54.73	GT
REF								
CP	0	39	59	62	69	69	69	77

KEY
0.0-57.53% - % wt. losses of infected wood blocks

TABLE 5.3.

PERCENTAGE SIMILARITY INDEX FOR A RANGE OF *C. PUTEANA* FPRL 11E INFECTED WOOD BLOCKS NOTED BY PERCENTAGE WEIGHT LOSSES

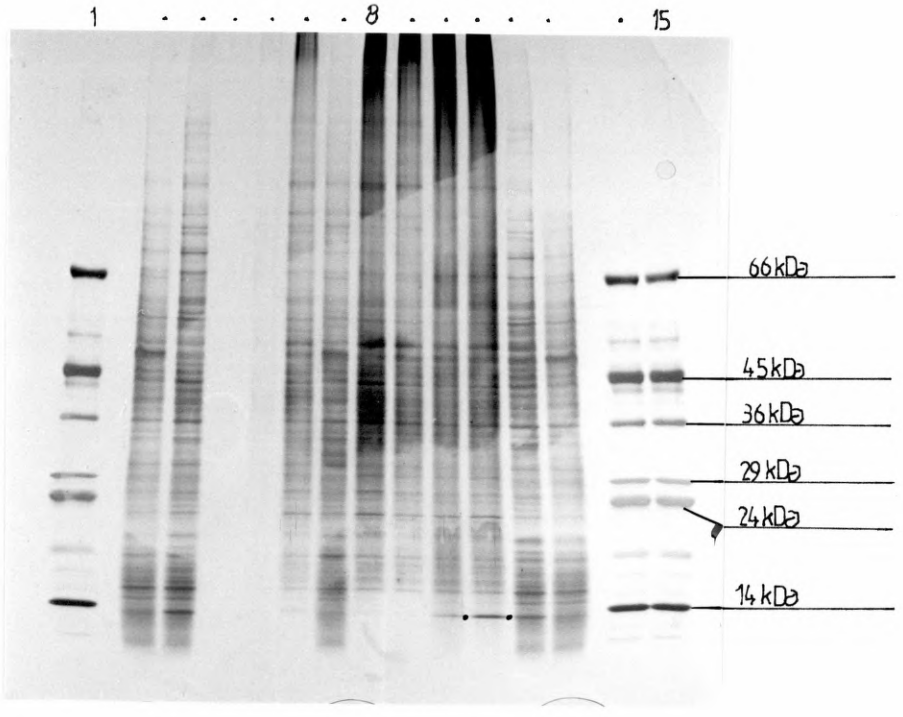


FIGURE 5.1. SDS-PAGE analysis of *C. puteana* FPRL 11E infected wood blocks. Tracks represent: 1, 14, 15 - Standard molecular weight markers (MWM); 2, 7, 13 - *C. puteana* FPRL 11E; 3, 12 - GT extract of *C. puteana* FPRL 11E. The following tracks represent various percentage weight losses of infected wood blocks: 4 - uninfected; 5 - 0.92%; 6 - 16.74%; 8 - 26.48%; 9 - 34.12%; 10 - 49.15%; 11 - 54.73%. In this and all subsequent figures the molecular weight markers used were of the following sizes, 14,200, 20,000, 24,000, 29,000, 36,000, 45,000 and 66,000 Daltons.

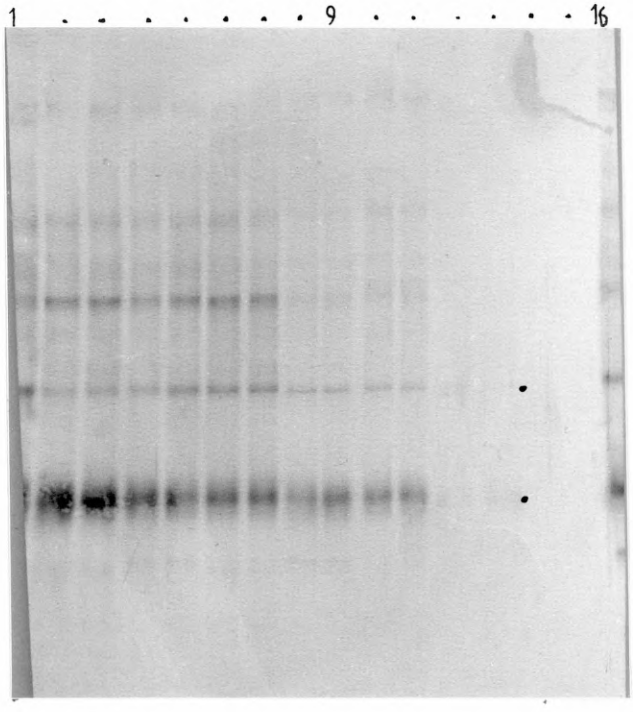


FIGURE 5.2. Western blotting analysis of *C. puteana* FPRL 11E infected wood blocks. Tracks represent: 1, 16 - *C. puteana* FPRL 11E: The following tracks represent various percentage weight losses of infected wood blocks. 2, 3 - 54.73%; 4, 5 - 49.15%; 6, 7 - 34.12%; 8, 9 - 26.48%; 10, 11 - 16.74%; 12, 13 - 0.92%; 14, 15 - uninfected.

there were no other specific changes within the protein profiles of the wood extracts. The 14,200 Da protein is evident in both the protein profiles of the standard and GT extracts, however, this protein band is more intense in the profile of the GT extract (tracks 3, 12). The numerical analysis shown in Table 5.3. also confirms the close similarity between the profile of the GT extract (tracks 3, 12) and the profile of the standard extract (tracks 2, 7, 13).

Results obtained for the antigen analysis of extracts of *C. puteana* FPRL 11E infected wood blocks, with antiserum 88/1 (Figure 5.2.) also indicated a similarity between the antigen profiles of the infected wood extracts and that of the FPRL 11E profile. A faint antigenic signal is evident in the western blot, for a wood block with a weight loss of 0.92% (tracks 12, 13) indicating the detection of antigens of *Coniophora*. In addition some evidence to support the identity of these antigens as specific antigens of *C. puteana* is apparent (Table 5.4.). They represent antigens 3 and 4 of the main antigens present in the standard profile of FPRL 11E.

5.2.2. COMPARISON OF PROTEIN/ANTIGEN PROFILES OF MORPHOLOGICAL REGIONS OF *C. PUTEANA* WITH THOSE OF INFECTED WOOD BLOCKS

The results presented in section 4.6., indicated that there were only minor differences between the protein profiles of the standard extract of FPRL 11E and the GT, intermediate and aged extracts of the same organism. An examination of the protein profiles of extracts of FPRL 11E infected wood blocks at low and high weight loss (16.74% and 54.73%) and

TEST	0.0	0.92	16.74	26.48	34.12	40.0	54.73
REF CP							
1*			*	*	*	*	*
2*			*	*	*	*	*
3*		*	*	*	*	*	*
4*		*	*	*	*	*	*
5*			*	*	*	*	*

KEY

* - Antigens common to FPRL 11E

0.0-54.73% - % wt. losses of infected wood blocks

TABLE 5.4.

ANALYSIS FOR COMMON ANTIGENS OF *C. PUTEANA* INFECTED WOOD BLOCKS TO FPRL 11E

TEST / REF	CP	Lwb	Hwb	GT	INTER	AGED
CP	100	58	65	74	61	65
Lwb	72	100	92	76	56	68
Hwb	73	89	100	81	54	65

KEY

AGED	-	aged mycelia
INTER	-	intermediate mycelia
Lwb	-	low weight loss block
Hwb	-	high weight loss block

TABLE 5.5.

PERCENTAGE SIMILARITY INDEX FOR THE COMPARISON OF INFECTED WOOD BLOCKS TO THE MORPHOLOGICAL REGIONS OF *C. PUTEANA* MYCELIUM

the protein profiles of the 3 morphological regions of the fungal mycelium was undertaken (Figure 5.3.). A study to determine any similarities of specific proteins in the profiles of these extracts with the profiles of extracts of wood blocks infected by *C. puteana*, was carried out. With the exception of the 14,200 Da protein present in the profile of the GT extract (track 9) and in the high percentage weight loss block (tracks 5, 8, 11 and Figure 5.1.), there were no specific similarities evident. However the numerical analysis of the profiles (Table 5.5.) did reveal a greater parity between the extracts of infected wood blocks and the GT extract (76% - Lwb; 81% - Hwb) than that observed for the comparison of the wood blocks with the other 2 regions of the fungal mycelium (56%, 68% - Lwb; 54%, 65% - Hwb) and to the standard FPRL 11E (72% - Lwb; 73% - Hwb). In addition the profiles of both infected blocks more closely resembled the profile of the whole mycelium of *C. puteana*, than the intermediate and aged profiles.

The antigen profiles produced by extracts of, a) the 3 morphological regions of the fungal mycelium and b) low and high percentage weight loss blocks when analysed using antiserum 88/1 (data not shown) were substantially similar to the standard profile of FPRL 11E which is shown in Figure 5.2. Each extract exhibited the 5 major antigens of FPRL 11E. A further analysis of these extracts using an alternative antiserum (90/2, raised against GT) was undertaken (Figure 5.4.). The antigens detected in the profile for the wood extracts (tracks 4, 5, 7, 8, 10, 11) are the same antigens detected for the comparative extracts (GT, intermediate and aged mycelia; tracks 6, 9, 3, 12). The approximate molecular weights of the 2 main antigens

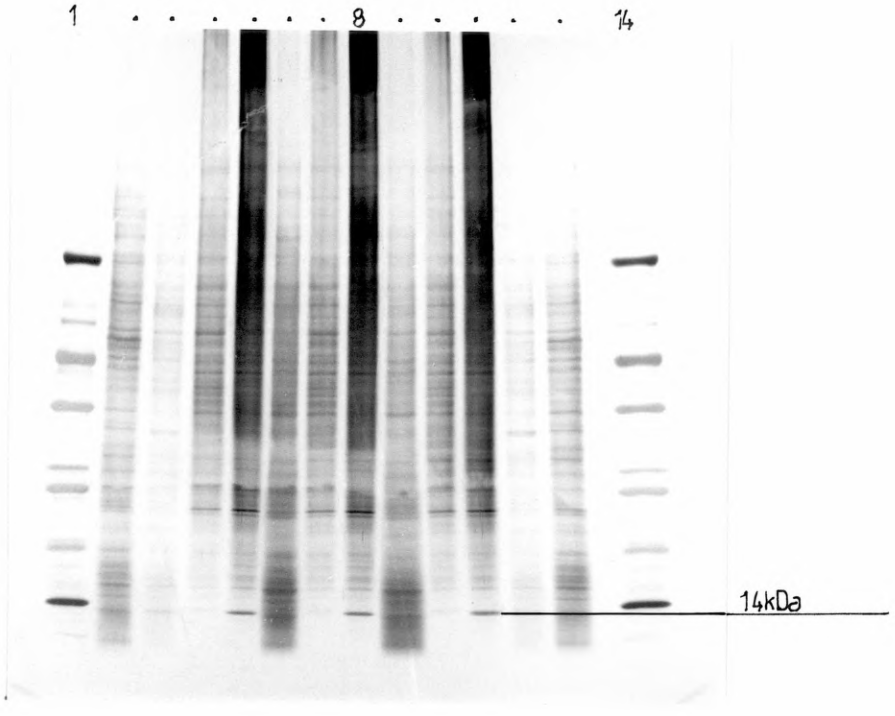


FIGURE 5.3. SDS-PAGE analysis of *C. puteana* FPRL 11E infected wood blocks and extracts of the 3 morphological regions of the fungal mycelium. Tracks represent: 1, 14 - MWM (Figure 5.1.); 2, 13 - *C. puteana* FPRL 11E; 3, 12 - aged mycelia; 4, 7, 10 - 16.74% weight loss block; 5, 8, 11 - 54.73% weight loss block; 6 - intermediate mycelia; 9 - GT.

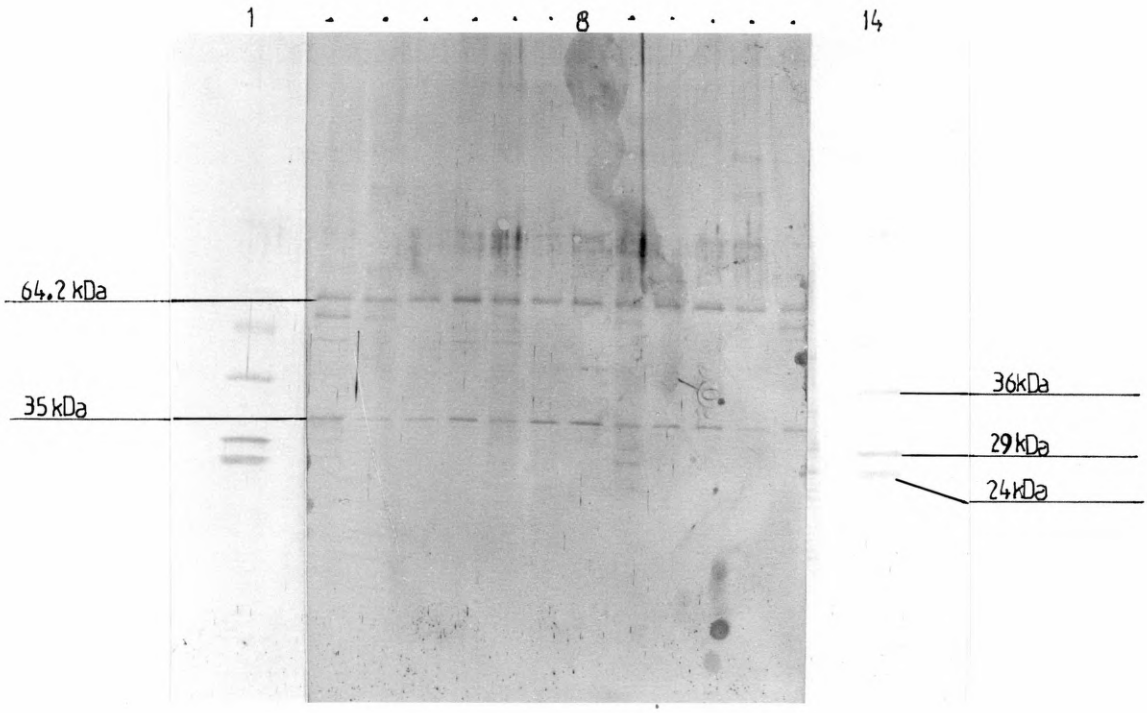


FIGURE 5.4. Western blotting analysis of *C. puteana* FPRL 11E infected wood blocks and extracts of the 3 morphological regions of the fungal mycelium, using antiserum 90/2. Tracks represent: 1, 14 - MWM (Figure 5.1.); 2, 13 - *C. puteana* FPRL 11E; 3, 12 - aged mycelia; 4, 7, 10 - 54.73% weight loss block; 5, 8, 11 - 34.12% weight loss block; 6 - GT; 9 - intermediate mycelia.

observed in all extracts are 64,200 Da and 35,000 Da respectively as indicated in Figure 5.4. and may correspond to the more diffuse antigens 3 and 4 detected by antiserum 88/1, viz., 68,400-65,300 Da and 32,000 Da (3.4.1. and Figure 5.2).

5.3. THE EFFECT OF DESICCATION ON THE PROTEIN/ANTIGEN PROFILES OF *C. PUTEANA* IN WOOD

The remedial treatment for the eradication of *C. puteana* infections and indeed infections by all wood decay fungi involves 2 important stages. The first involves complete drying of the wood, to prevent further growth of the organism, thus inhibiting further decay and the second involves the application of an appropriate preservative to kill the organism and protect the wood from any further attack by wood destroying fungi. Methodologies to monitor the effect of drying and preservatives are currently only possible using isolation studies, however these treatments might be associated with specific molecular changes within the fungal hyphae. Consequently an analysis was undertaken to investigate the affect of drying on the molecular profiles of *C. puteana*.

Wood blocks decayed for 6 weeks were examined after periods of desiccation of 1, 2, 3 and 4 weeks (2.15.5., Figure 2.1.). The viability of *C. puteana* was tested at each of these intervals by the re-introduction of dried infected wood blocks into a nutrient and moisture rich environment (culture jars containing 2% A/5% MX medium). The results shown in Figure 5.5., indicate that after 3 and 4 weeks desiccation of the infected wood blocks, the protein profiles of extracts of these blocks are substantially

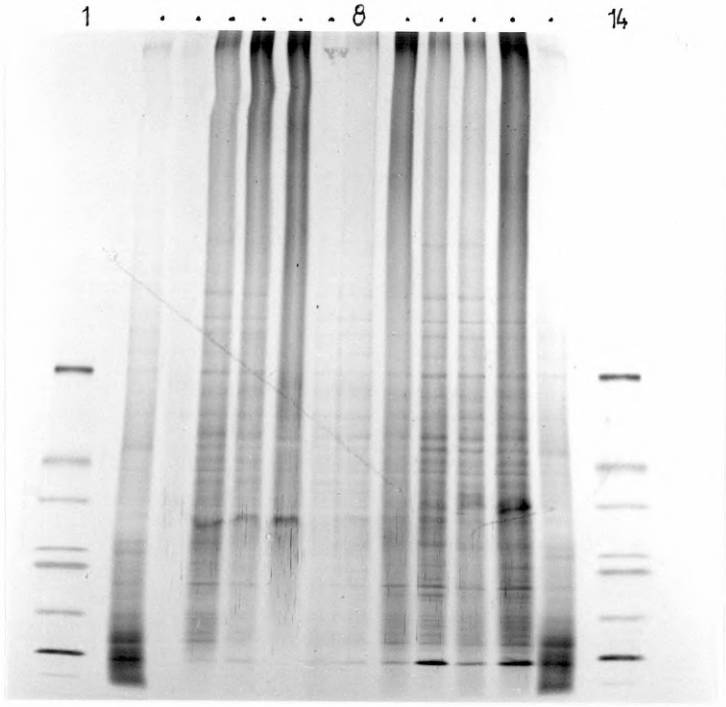


FIGURE 5.5. SDS-PAGE analysis of *C. puteana* FPRL 11E infected wood blocks subjected to 1-4 weeks desiccation and subsequently re-introduced into a nutrient and moisture rich environment (further decay). Tracks represent: 1, 14 - MWM (Figure 5.1.); 2, 13 - *C. puteana* FPRL 11E; 3 - uninfected wood block; 4 - 16.74% weight loss block; 5, 6, 7, 8 - infected wood blocks desiccated for 1, 2, 3, 4 weeks respectively; 9, 10, 11, 12 - wood blocks desiccated for 1-4 weeks respectively and subjected to further decay for 4 weeks.

similar to the profile observed for an extract of a block at 34.12% weight loss (track 4) and that observed for FPRL 11E (track 2), the fainter track being due to a concentration effect in the experiment. Tracks 9-12 indicate that on re-introduction into a nutrient and moisture rich environment a profile which is substantially similar to that of *C. puteana* is evident (track 2), and shows no specific changes in individual proteins. After 4 weeks drying, *C. puteana* was still viable and its molecular integrity intact. Results for an antigen analysis using antiserum 88/1 of *C. puteana* grown in wood (Figure 5.6.) indicated that the antigen profiles were essentially the same as previously reported. One extra antigen was found in the infected wood blocks in this experiment and was present in both control and experimental blocks. The similarities within the antigen profiles of the infected wood blocks, suggests that drying does not affect the antigenic nature of *C. puteana* grown in wood.

The experiment described above which used a relatively short drying period of up to 4 weeks, indicated that drying caused no specific changes in the protein and antigen profile of *C. puteana*. The effect of an extended drying period was examined. Blocks were decayed for 6 weeks and subjected to desiccation for 3-12 weeks. At 3, 6, 9 and 12 weeks extracts of selected blocks were examined by SDS-PAGE and western blotting, whilst the remaining blocks were introduced into an agar jar for 4 weeks for *C. puteana* viability testing (2.15.5., Figure 2.2.). The results for the consequent weight losses of blocks are shown in Table 5.6. Consistent results were obtained for blocks desiccated for 3 and 6 weeks, in that averages of 61-65% were obtained even after the blocks were subjected to further decay.

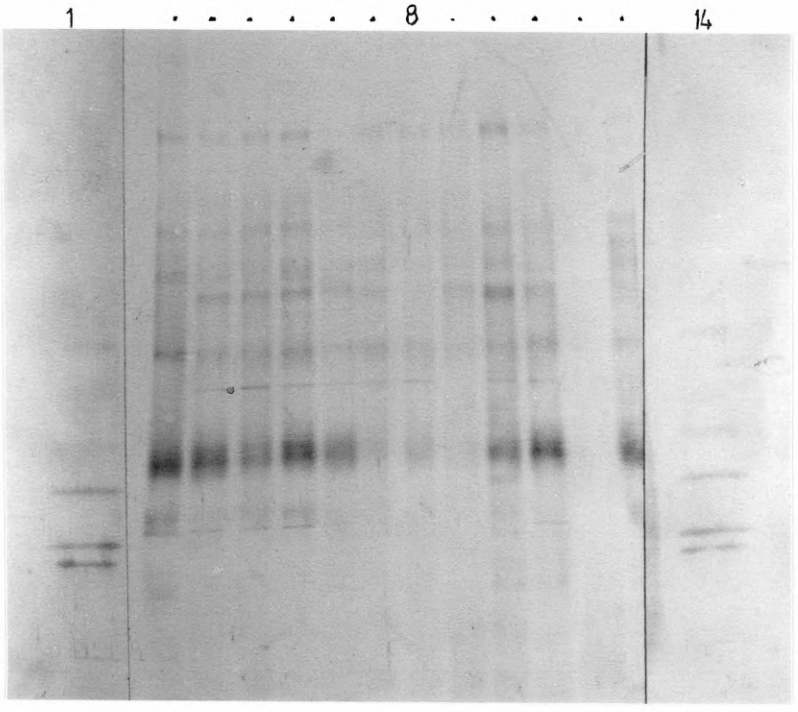


FIGURE 5.6. Western blotting analysis of *C. puteana* FPRL 11E infected wood blocks subjected to 1-4 weeks desiccation and subsequently re-introduced into a nutrient and moisture rich environment (further decay). Tracks represent: 1, 14 - MWM (Figure 5.1.); 2, 13 - *C. puteana* FPRL 11E; 3, 4, 5, 6 - wood blocks desiccated for 4, 3, 2 and 1 week(s) respectively and subjected to further decay for 4 weeks; 7, 8, 9, 10 - infected wood blocks desiccated for 4, 3, 2 and 1 week(s) respectively; 11 - 34.12% weight loss block; 12 - uninfected wood block.

TIME PERIOD OF DESICCATION	PERCENTAGE WEIGHT LOSS PER WOOD BLOCK			
	>DESICCATION		>DECAY FOR 4 WKS	
3 WKS	NO.	% WT. LOSS	NO.	% WT. LOSS
	25	64.40	26	63.27
	30	66.75	27	63.55
	32	60.75	28	62.59
	50	62.59	29	66.32
	52	62.72	31	51.16
	Av	63.44	Av	61.38
6 WKS	36	64.55	33	16.13
	40	60.37	35	60.20
	44	65.18	38	64.47
	93	61.92	39	63.24
	34	70.73	47	63.17
	Av	64.55	Av	62.77
9 WKS	41	11.98*	46	55.17*
	42	9.63*	48	27.85*
	43	7.50*	4	28.42*
	45	9.50*	5	16.91*
	35	65.98	96	63.82
12 WKS	55	43.70*	53	43.04*
	49	41.14*	56	40.53*
	61	10.20*	59	50.31*
	60	10.31*	62	53.51*
	24	63.38	63	58.28

KEY
Av - Average
WKS - Weeks
* - Anomalous % weight losses
NO - Individual number for each wood block
> - After desiccation or decay.

NB. All blocks were decayed for 8 wks prior to desiccation. For further details of methodology see 2.15.5.

TABLE 5.6.

PERCENTAGE WEIGHT LOSSES OBTAINED FOR BLOCKS SUBJECTED TO DESICCATION AND FURTHER DECAY

Since the cellulose and hemicellulose content of wood are 41% and 31% respectively (Glancy, 1990), these consistently high weight losses may represent highly degraded wood blocks thus only very limited additional decay is possible. The high weight losses observed, were unexpected, since the previous experiment suggested that weight losses after the same period of decay would be no greater than 35% (data not shown). Consequently it is not possible using these weight loss results to determine whether *C. puteana* has been affected by desiccation. However evidence that drying had no substantial effect on the viability of *C. puteana* is provided by the fact that it was possible to re-isolate the organism from the agar cultures, even after 6 weeks desiccation.

The results for blocks desiccated for 9 and 12 weeks exhibited a variation in percentage weight loss values. The high percentage weight loss values of 65.98% and 63.82% (9 weeks), and 63.38% and 58.28% (12 weeks), were expected results and are consistent with weight losses observed for weeks 3 and 6. However lower weight loss values were also apparent, the very low values (7-12%) perhaps indicating that these blocks were only latterly infected due to waterlogging at the initiation of the decay experiment. The remaining percentage weight loss values, which varied substantially from 10%-55% indicate that the method of decay used is not sufficient to provide reproducible results for wood decayed by *C. puteana*. The anomalous results which are indicated in Table 5.6., however may mimic the decay of wood by *C. puteana* in an uncontrolled environment as would occur in the field situation. That *C. puteana* was still viable after 12 weeks desiccation was

noted by the re-isolation of the organism from the agar cultures.

Initially, the experiment to determine the effect of desiccation on *C. puteana* was designed to detect any changes in specific proteins and antigens which may be essential for the growth of the organism. In addition since 2 sets of results were apparent, a)consistently highly degraded blocks and b) anomalous blocks (which may reflect decay in an uncontrolled environment), a comparative analysis of these blocks using SDS-PAGE, was carried out. The appearance or disappearance of specific proteins after desiccation was noted. The selected wood blocks for this analysis are shown in Table 5.7.

Results of the protein analysis of anomalous percentage weight loss blocks are shown in Figure 5.7. There was a lack of distinction of bands in some samples due to black streaking. This artifact is probably a consequence of the very acidic pH of the high percentage weight loss samples. Attempts to reduce this streaking by the addition of boiling mix and 0.1M sodium hydroxide to increase the pH were unsuccessful. Attempts to reduce the affect by diluting the samples were also unsuccessful, as it was often the case that the individual protein bands were now too faint to be observed. The gels exhibited are those containing the most satisfactory profiles. Despite these problems, consistent banding profiles could be seen in some instances (Figure 5.7. tracks 6, 7, 10, 11, 12), even if they were sometimes partially obscured by the streaking effect.

TIME PERIOD OF DESICCATION	PERCENTAGE WEIGHT LOSS PER WOOD BLOCK			
	HIGHLY DEGRADED BLOCKS		ANOMALOUS BLOCKS	
	>DESICCATION	>DECAY	>DESICCATION	>DECAY
0 WKS*				
3 WKS	30 - 66.75	29 - 66.32	32 - 60.75	28 -62.59
6 WKS	34 - 70.73	38 - 64.47	40 - 60.37	35 - 60.20
9 WKS	35 - 65.98	96 - 63.82	43 - 7.5	5 - 16.91
12 WKS	24 - 63.38	63 - 58.28	61 - 10.20	56 - 40.53

TABLE 5.7.

DEGRADED AND ANOMALOUS BLOCKS SELECTED FROM RESULTS SHOWN IN TABLE 5.6. FOR USE IN SUBSEQUENT ANALYSES

KEY

WKS - Weeks

* - WK0, Highly degraded block no. 54 (42.45%) and anomalous block no. 6 (3.12%) were used in subsequent analyses.

> - After desiccation or decay.

NB All numbers before, -, are numbers of individual wood blocks.

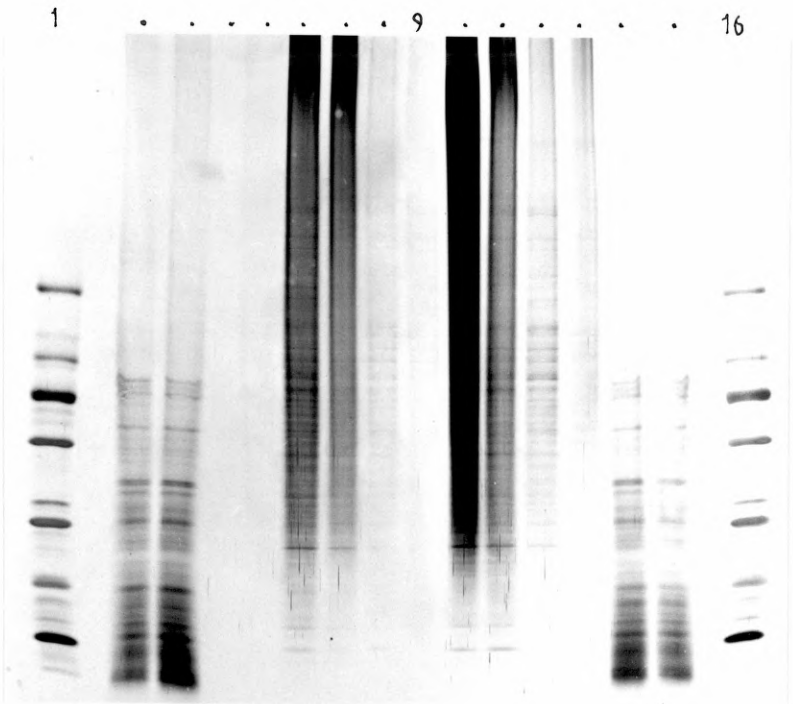


FIGURE 5.7. SDS-PAGE analysis of *C. puteana* FPRL 11E infected wood blocks subjected to 3-12 weeks desiccation and subsequently re-introduced into a nutrient and moisture rich environment (Anomalous weight losses). Tracks represent: 1, 16 - MWM (Figure 5.1.); 2, 15 - *C. puteana* FPRL 11E; 3, 14 - *C. puteana* grown from wood blocks after 12 weeks desiccation; 4 - uninfected wood block; 5 - 3.12% weight loss block; 6, 7, 8, 9 - infected wood blocks desiccated for 3, 6, 9, 12 weeks respectively (Table 5.7.); 10, 11, 12, 13 - wood blocks desiccated for 3-12 weeks respectively and subjected to further decay for 4 weeks (Table 5.7.).

Similarities between the profiles of extracts of the infected wood samples to that of FPRL 11E (tracks 2, 15) are evident although minor differences are apparent confirming previous analysis of wood infected material (5.2.1., Figure 5.1.). Although the intensities of the protein bands within the profiles of the wood extracts become lighter, no changes in specific proteins are evident. An analysis of the protein profile of *C. puteana* isolated after 12 weeks desiccation (tracks 3, 14) indicated that the profile was exactly similar to that of FPRL 11E (tracks 2, 15).

Figure 5.8., shows the protein profiles obtained from the analysis of the highly degraded blocks (Table 5.7.). The protein profiles of extracts of these *C. puteana* infected wood blocks are substantially similar to the profile of FPRL 11E (tracks 2, 15) although the black streaking rendered the analysis difficult. After 12 weeks, re-isolated mycelia of *C. puteana* (tracks 3, 14) produced a protein profile which was substantially similar to *C. puteana* FPRL 11E (tracks 2, 15).

Further evidence to support the conclusion that the molecular nature of *C. puteana* is not affected by desiccation was provided by the production of antigenic profiles of the highly degraded blocks using antiserum 88/1 (Figure 5.9.). The profiles indicate that even after 12 weeks the antigen profile of the desiccated wood block (track 8) and that of the isolated *C. puteana* mycelia (tracks 3, 14) are similar to the profile of FPRL 11E (tracks 2, 15). Unlike SDS-PAGE the antigenic analysis was unaffected by the high acidity of the decayed blocks.

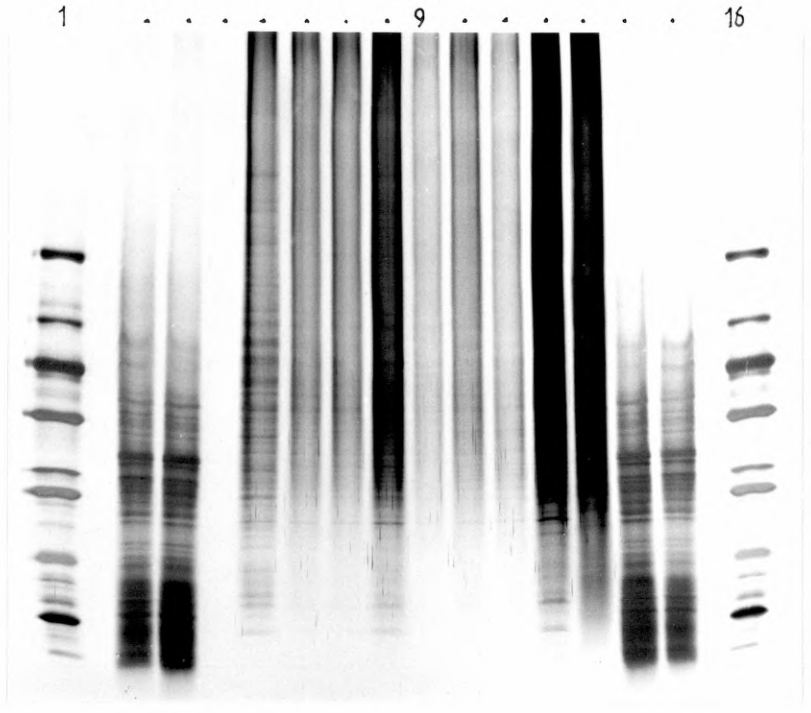


FIGURE 5.8. SDS-PAGE analysis of *C. puteana* FPRL 11E infected wood blocks subjected to 3-12 weeks desiccation and subsequently re-introduced into a nutrient and moisture rich environment (Totally degraded blocks). Tracks represent: 1, 16 - MWM (Figure 5.1.); 2, 15 - *C. puteana* FPRL 11E; 3, 14 - *C. puteana* grown from wood blocks after 12 weeks desiccation; 4 - uninfected wood block; 5 - 42.45% weight loss block; 6, 7, 8, 9 - infected wood blocks desiccated for 3, 6, 9, 12 weeks respectively (Table 5.7.); 10, 11, 12, 13 - wood blocks desiccated for 3-12 weeks respectively and subjected to further decay for 4 weeks (Table 5.7.).

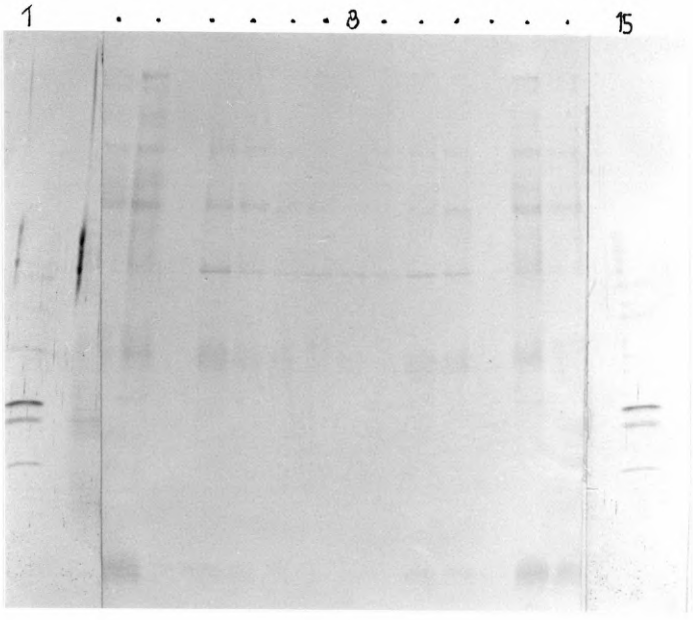


FIGURE 5.9. Western blotting analysis of *C. puteana* FPRL 11E infected wood blocks subjected to 3-12 weeks desiccation and subsequently re-introduced into a nutrient and moisture rich environment (Totally degraded blocks; antiserum 88/1). Tracks represent: 1, 15 - MWM (Figure 5.1.); 2, 14 - *C. puteana* FPRL 11E; 3 - *C. puteana* grown from wood blocks after 12 weeks desiccation; 4, 5, 6, 7 - wood blocks desiccated for 12, 9, 6 and 3 weeks respectively and subjected to further decay for 4 weeks (Table 5.7.); 8, 9, 10, 11 - infected wood blocks desiccated for 12, 9, 6 and 3 weeks respectively (Table 5.7.); 12 - uninfected wood block; 13 - 42.45% weight loss block.

5.4. THE DETECTION OF *C. PUTEANA* IN WOOD BLOCKS BY MOLECULAR AND IMMUNOLOGICAL ANALYSIS USING POLYCLONAL ANTISERA

Due to the potential of immunological methods as simple identification and detection systems, the following analysis deals with the detection of *C. puteana* in wood by western blotting using various polyclonal antisera. Subsequent detection of the organism using the simpler dot immunoblotting and EIA techniques was carried out using the same antisera.

5.4.1. DETECTION OF *C. PUTEANA* USING SDS-PAGE AND WESTERN BLOTTING

In order to analyse the antigenicity of *C. puteana* in wood samples, western blotting using the 3 different immunological reagents described in chapter 4, viz., 88/1, 88/8 and 90/2, raised against WM extracts, exoprotein extracts and GT extracts respectively, was carried out. Although in previous analyses these antisera have been shown to be highly cross-reactive with other fungi known to inhabit wood, it was necessary to determine their ability to detect *C. puteana* at all stages of decay in order to further develop suitable immunological reagents for the detection of *C. puteana*.

The detection of a recognisable protein profile of *C. puteana* using SDS-PAGE was possible at weight losses at or greater than 26.48% (5.2.1., Figure 5.1.). Antigenic detection of *C. puteana* using western blotting with antiserum 88/1 was however possible at weight losses of 16.74% or greater and possibly at even lower weight losses

(5.2.1., Figure 5.2.). Antigenic detection of *C. puteana* decayed wood blocks is also possible with antisera 88/8 and 90/2 (Figures 5.10. and 5.11. respectively), although antigens are not evident at 0.92% weight loss. The poor quality of the blot exhibited in Figure 5.10. is indicative of the difficulty of analysing exoantigens by this method. Some indication of the presence of exoantigens is evident at weight losses of 16.74% (track 10), or greater. However substantially clearer antigen profiles were obtained using antiserum 90/2 (Figure 5.11). The presence of an antigen profile at 16.74% weight loss or greater is evident and the profiles are substantially similar to those of the standard FPRL 11E (tracks 3, 9, 14) and to the GT profile (tracks 4, 13).

5.4.2. DETECTION OF *C. PUTEANA* IN WOOD USING DOT IMMUNOBLOTTING AND EIA

WM and EP preparations of a range of infected wood blocks were analysed by dot immunoblotting. However non-specific binding of the primary antisera to the uninfected wood block was evident and attempts to reduce this by pre-incubation of the antisera with uninfected sawdust proved unsuccessful, effectively preventing the use of this system to screen extracts from decayed blocks for the presence of *C. puteana*.

WM and EP preparations from a range of infected wood blocks were subsequently analysed by EIA using both 88/1 and 88/8 antisera and results are shown in Figures 5.12. and 5.13. respectively. Detection of *C. puteana* in decayed wood blocks is possible at all stages of decay using both antisera and both antigen extracts. Each antiserum reacts

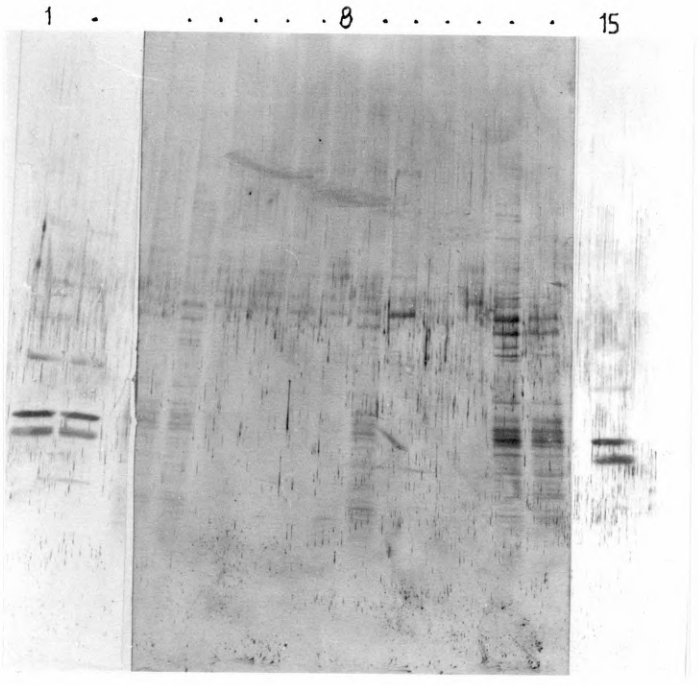


FIGURE 5.10. Western blotting analysis of *C. puteana* FPRL 11E infected wood blocks using antiserum 88/8. Tracks represent: 1, 2, 15 - MWM (Figure 5.1.); 3, 9, 14 - *C. puteana* FPRL 11E; 4, 13 - GT extract of *C. puteana* FPRL 11E. The following tracks represent various percentage weight losses of infected wood blocks; 5 - 54.73%; 6 - 49.15%; 7 - 34.12%; 8 - 26.48%; 10 - 16.74%; 11 - 0.92%; 12 - uninfected.

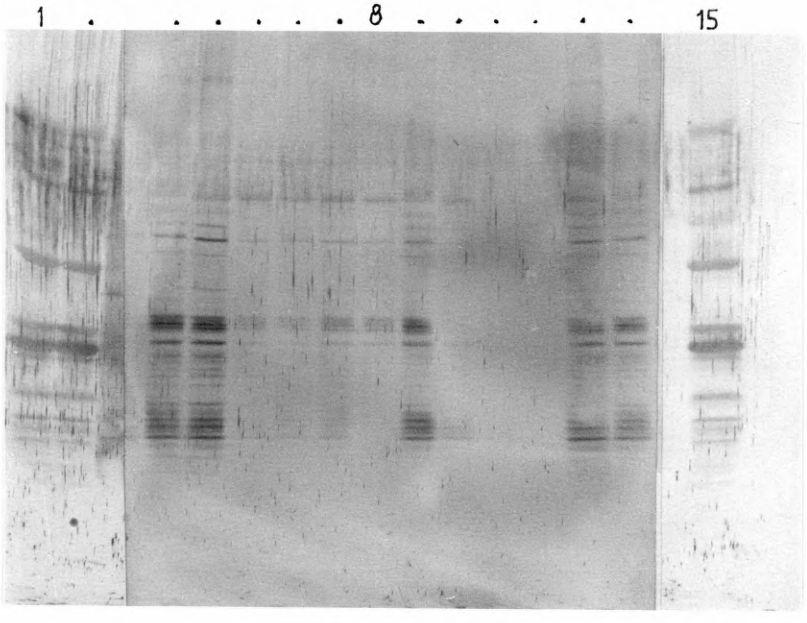
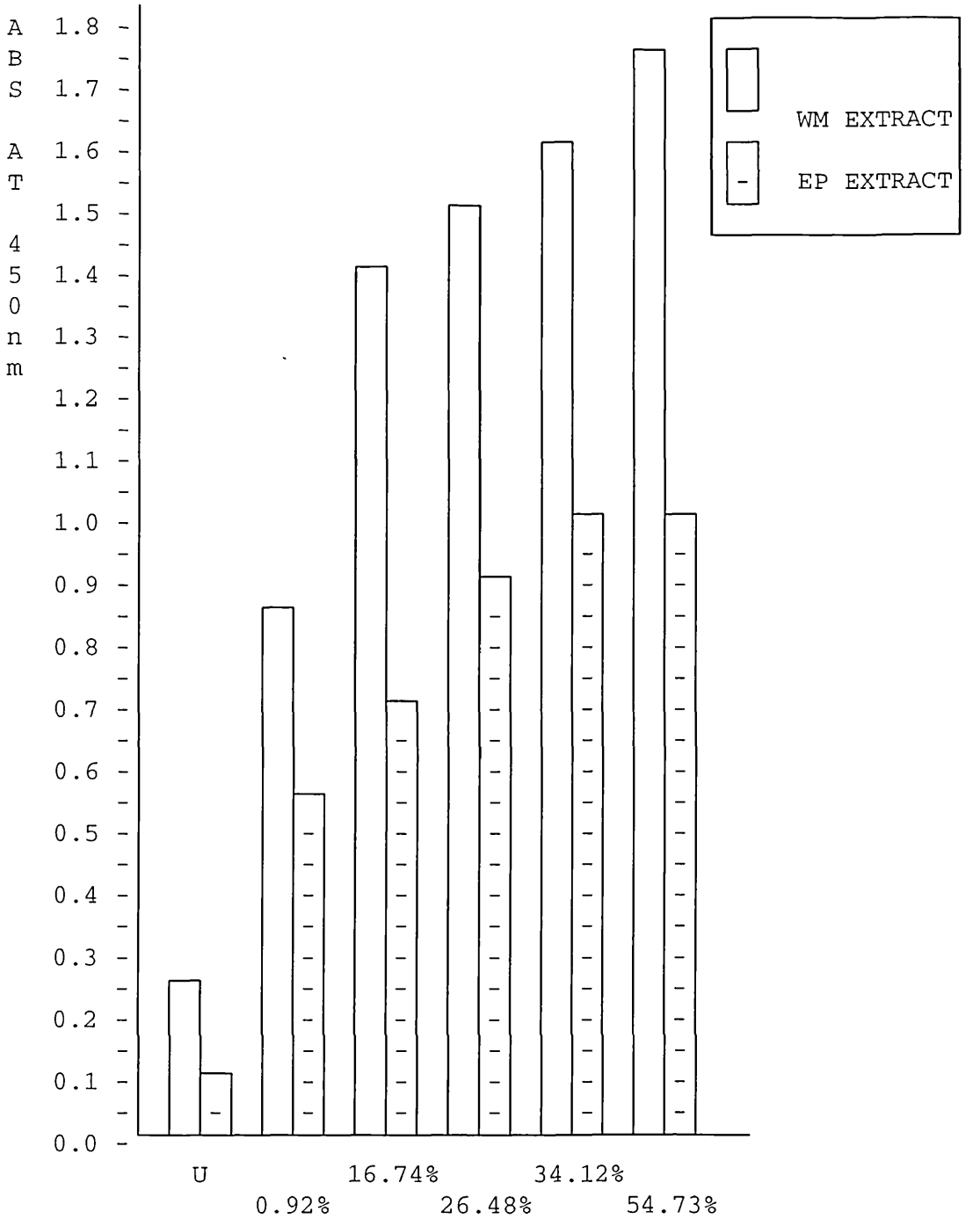


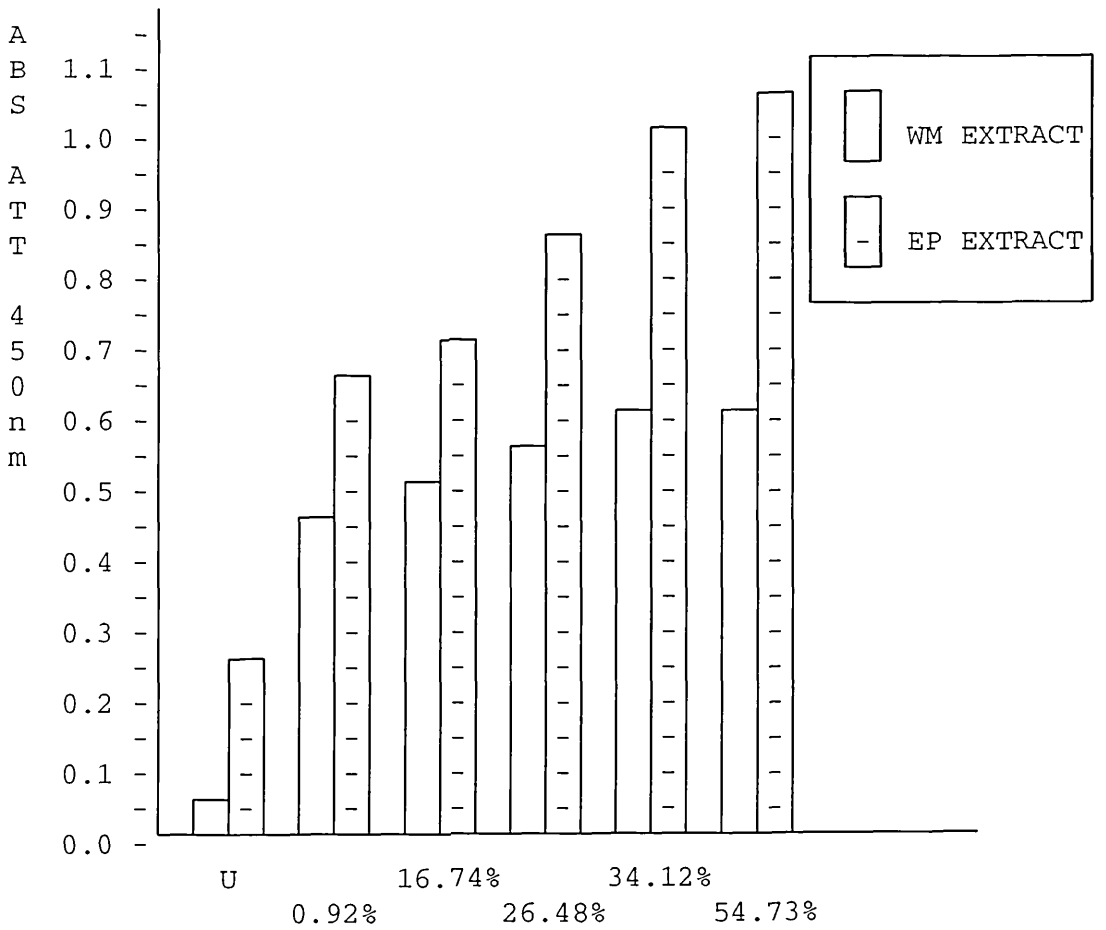
FIGURE 5.11. Western blotting analysis of *C. puteana* FPRL 11E infected wood blocks using antiserum 90/2. Tracks represent: 1, 2, 15 - MWM (Figure 5.1.); 3, 9, 14 - *C. puteana* FPRL 11E; 4, 13 - GT extract of *C. puteana* FPRL 11E. The following tracks represent various percentage weight losses of infected wood blocks; 5 - 54.73%; 6 - 49.15%; 7 - 34.12%; 8 - 26.48%; 10 - 16.74%; 11 - 0.92%; 12 - uninfected.



PERCENTAGE WEIGHT LOSSES OF WOOD BLOCKS

FIGURE 5.12.

EIA ANALYSIS OF EXTRACTS (x5 DILUTION) OF A RANGE OF *C. PUTEANA* FPRL 11E INFECTED WOOD BLOCKS, WITH ANTISERUM 88/1



PERCENTAGE WEIGHT LOSSES OF WOOD BLOCKS

FIGURE 5.13.

EIA ANALYSIS OF EXTRACTS (x5 DILUTION) OF A RANGE OF *C. PUTEANA* FPRL 11E INFECTED WOOD BLOCKS, WITH ANTISERUM 88/8

to greater extent with its own immunogen, and antiserum 88/1 reacting with WM extracts of wood blocks infected with *C. puteana* produces the greatest signal. Each combination of antisera and immunogen can differentiate between an uninfected wood block and a block infected by *C. puteana* with a weight loss as low as 0.92%. Also each reagent has a similar ability to discriminate between different levels of decay.

5.5. DETECTION OF *C. PUTEANA* USING MONOCLONAL ANTIBODIES

5.5.1. INITIAL ANALYSIS OF ANTISERA

Various polyclonal antisera were produced in BALB-C mice to determine the most suitable immunogen for the production of monoclonal antibodies (MAbs). Antisera to EP extracts of infected wood blocks produced limited immune responses and the analysis of other EP extracts (Table 3.8.) indicated that EP4 would be the most suitable immunogen. The standard preparation of WM extract also produced satisfactory immune responses. EP4 was however initially selected for use in the production of MAbs (see discussion). The antibody dilution curves for the antisera produced by this immunogen are shown in Figure 5.14. Mouse 91/1a was selected for use in the fusion protocol for the production of MAbs.

5.5.2. SCREENING AND CROSS-REACTIVITY OF HYBRID COLONIES

Using the spleen from mouse 91/1a, 522 of 618 wells (84.5%) contained 1 or more hybrid colonies in a fusion experiment. Six percent of the hybrids were positive for antibody production against *C. puteana* FPRL 11E (33 from 522 possible positives)leven of the hybrids which were

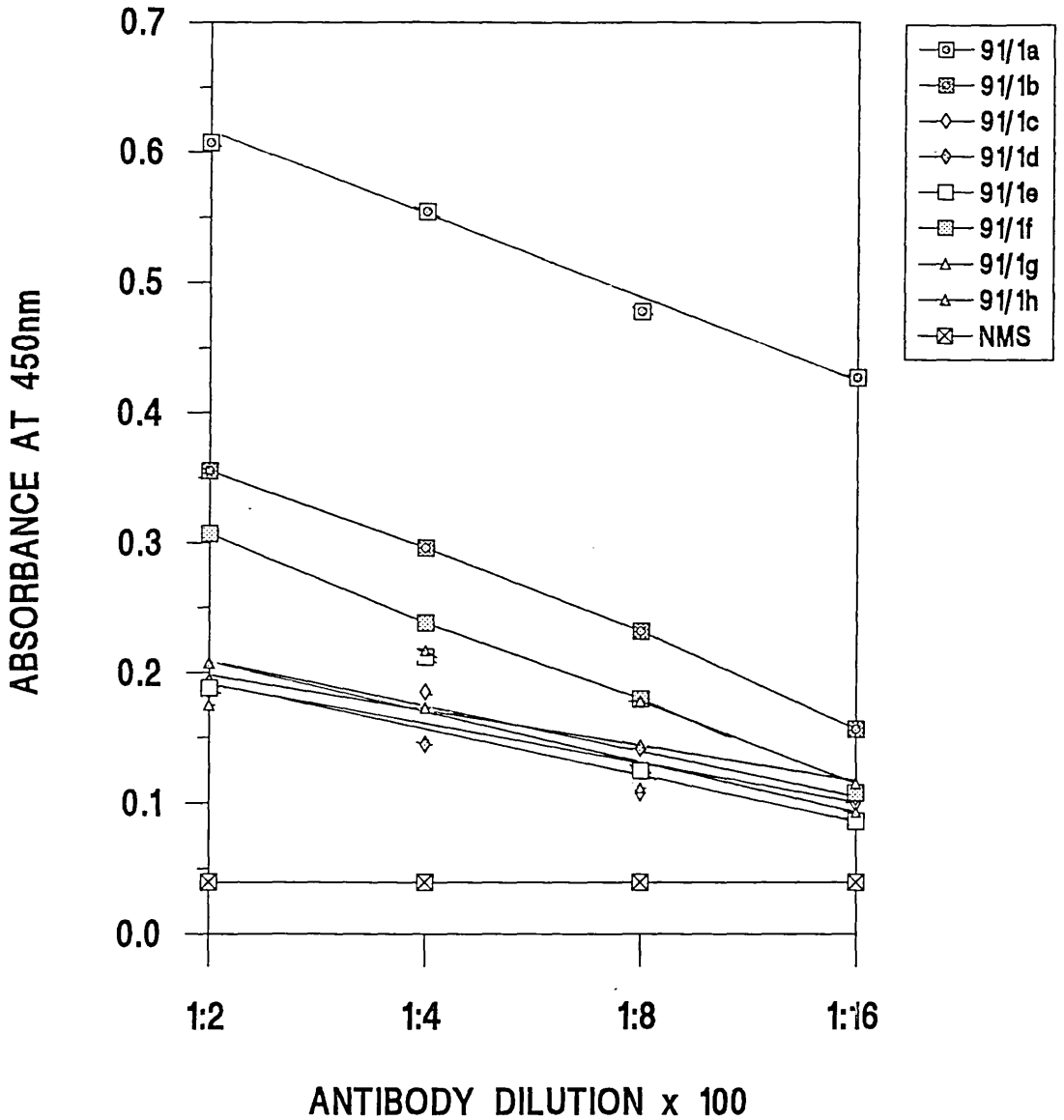


FIGURE 5.14. ANTIBODY DILUTION CURVE FOR ANTISERA 91/1(a-h)

positive for MAb production against *C. puteana* were cloned (Table 5.8.). The MAbs produced by the clones were tested for cross-reactivity with a range of basidiomycetes some of which are known to inhabit the timbers of the frigate *Unicorn* and RRS *Discovery* (Table 6.6.). Eight of the 11 MAbs showed a high degree of specificity in that they reacted with all members of the genus *Coniophora* tested (Table 5.9.), but not with any of the *Unicorn* and *Discovery* isolates. Due to the high level of reactivity of MAb 91/5B6 (22) and its specificity to the genus *Coniophora*, MAb 91/5B6 was produced in large quantities for the detection of *C. puteana* in wood. The isotype of MAb 91/5B6 is Immunoglobulin M, sub-group Kappa (IgMk). The remaining 3 MAbs (11, 23 and 56), cross-reacted with fungi other than those of the genus *Coniophora*, viz., *S. lacrymans* and *A. xantha* (Figure 5.15.).

5.5.3. DETECTION OF *C. PUTEANA* IN WOOD USING MAb 91/5B6

WM and EP extracts of a range of infected wood blocks were analysed by EIA using MAb 91/5B6 (Figure 5.16.). The MAb was capable of detecting *C. puteana* FPRL 11E at weight losses at or greater than 0.92% with both antigen preparations. Although the MAb was produced against EP extracts of *C. puteana*, it was capable of detecting both extracts (WM and EP) and had a greater ability to detect the WM preparation of the organism. In comparison to the polyclonal antisera 88/1 and 88/8, there was no reaction of the MAb with extracts of uninfected wood and the MAb had a greater ability to distinguish between different levels of decay particularly between low percentage weight losses (Figure 5.16.).

CLONES	REFERENCE NUMBER FOR SUBSEQUENT TABLES
91/5G8	2
91/2F5	11
91/4B3	12
91/10D7	13
91/5B6	22
91/2B11	23
91/7G5	24
91/3B6	34
91/3F3	56
91/2F8	72
91/7C7	100

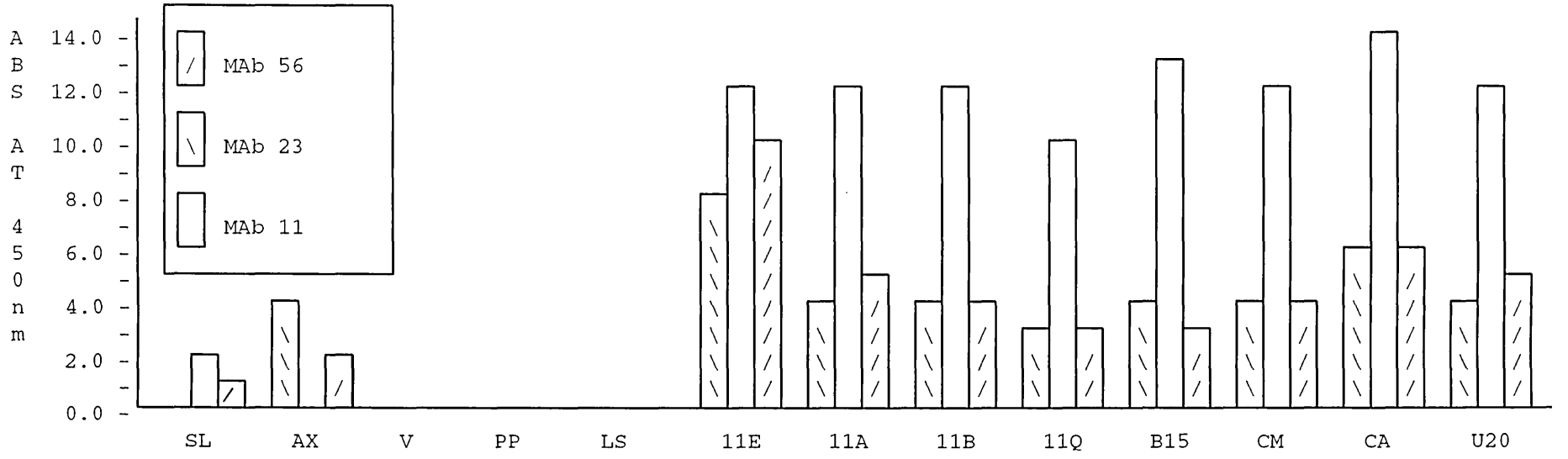
TABLE 5.8.

CLONES WHICH PRODUCED A POSTIVE REACTION WITH *C. PUTEANA* FPRL 11E

CONIOPHORA GENUS	MONOCLONAL ANTIBODIES							
	2	100	12	13	22	24	34	72
11E	3.9	4.3	2.9	1.4	5.4	3.5	2.7	2.1
11A	3.7	5.6	4.3	3.2	7.2	4.6	3.5	3.4
11B	3.2	5.1	8.1	4.9	5.8	5.0	3.6	3.8
11Q	3.1	4.3	3.2	2.6	5.9	4.0	2.5	2.5
B15	2.8	4.8	3.7	5.6	9.6	4.0	3.1	2.6
CM	2.9	4.0	3.4	2.0	5.7	3.6	2.3	2.0
CA	5.2	6.7	5.9	4.5	8.4	6.0	4.6	4.4
U20	3.3	4.8	3.8	2.3	5.7	4.4	3.3	2.6

TABLE 5.9.

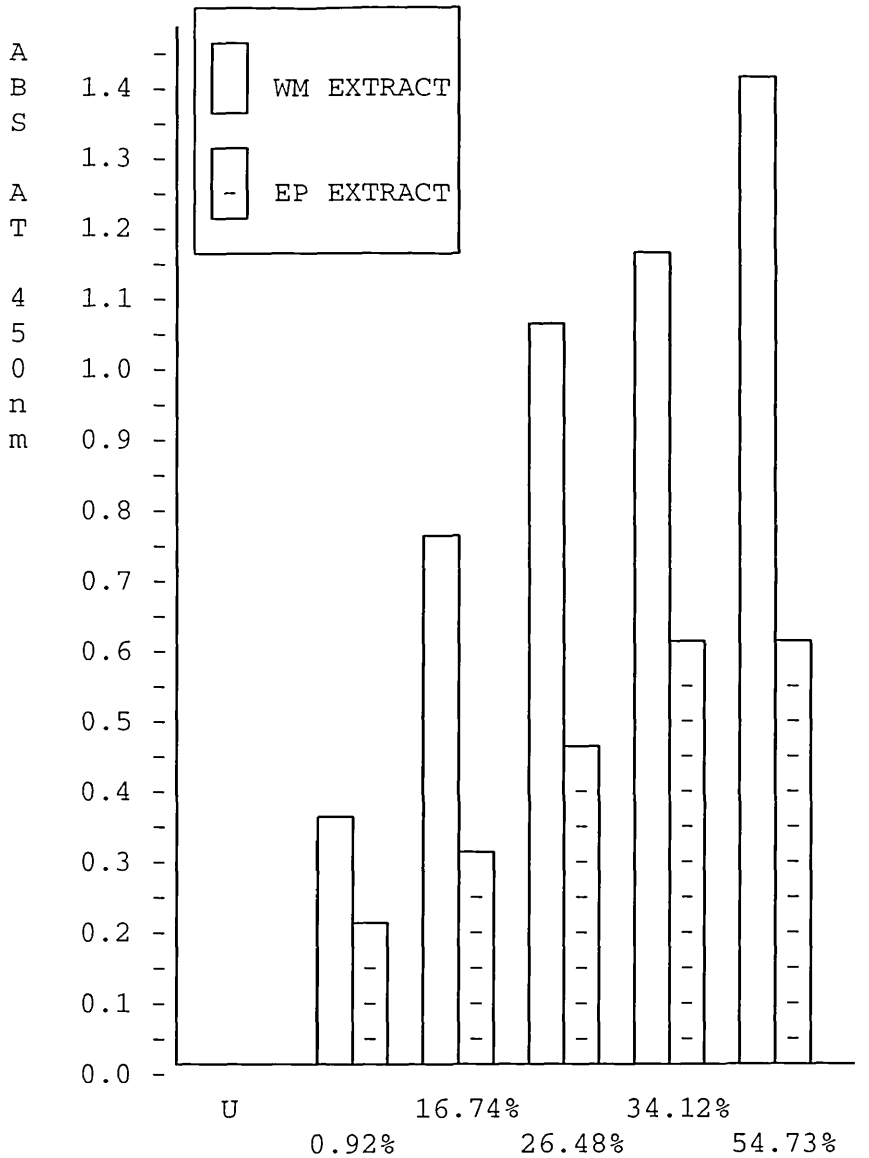
CROSS-REACTIVITY OF MONOCLONAL ANTIBODIES WITH MEMBERS OF THE GENUS *CONIOPHORA*



KEY

SL	- *S. lacrymans*
AX	- *A. xantha*
V	- *Verticillium*
PP	- *P. placenta*
LS	- *L. sulphureus*
11E-B15	- strains of *C. puteana*
CM	- *C. marmorata*
CA	- *C. arida*
U20	- isolate U20

FIGURE 5.15. CROSS-REACTIVITY OF 3 MAbs WITH BASIDIOMYCETES AND MEMBERS OF THE GENUS *CONIOPHORA*



PERCENTAGE WEIGHT LOSSES OF WOOD BLOCKS

FIGURE 5.16.

EIA ANALYSIS OF EXTRACTS (x5 DILUTION) OF A RANGE OF *C. PUTEANA* FPRL 11E INFECTED WOOD BLOCKS WITH MAb 91/5B6

5.6. DISCUSSION

5.6.1. IDENTIFICATION OF *C. PUTEANA* IN WOOD

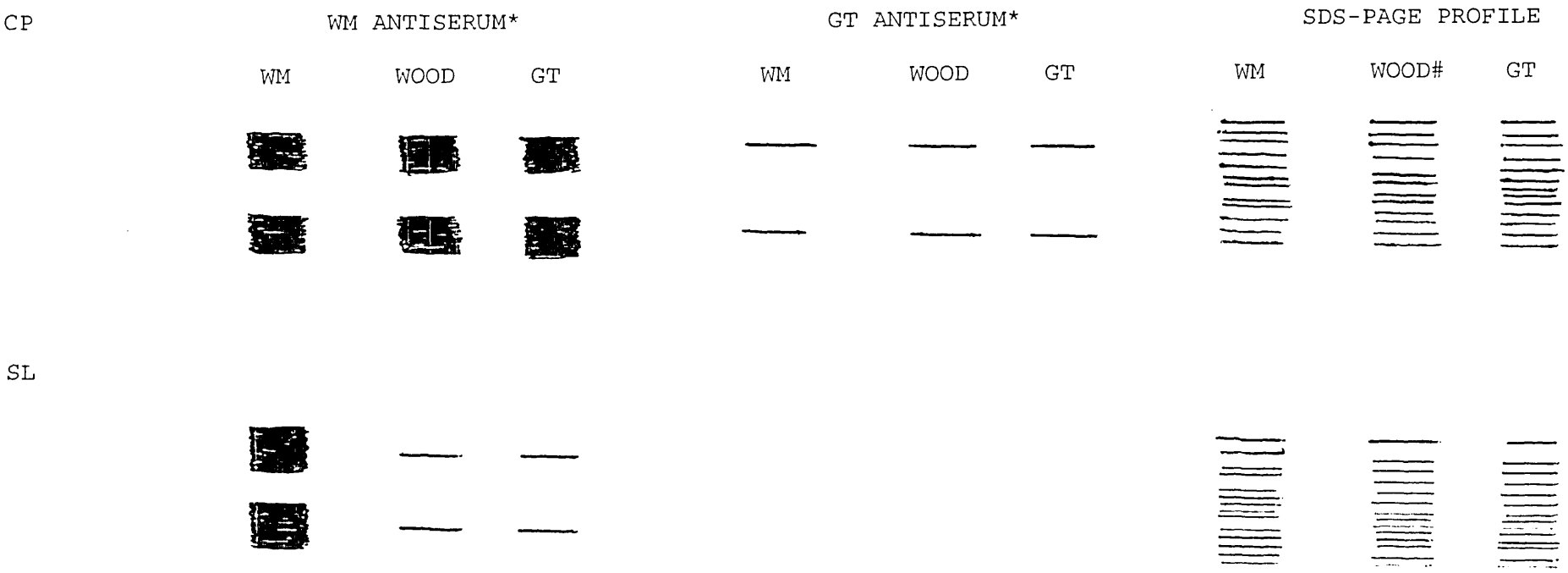
The research described in this chapter has indicated that both the protein and antigen profiles of extracts of *C. puteana* infected wood blocks are substantially similar to the profiles of extracts of the standard preparation of the organism, thus allowing a feasible system of identification in wood. Only 5 major antigens are detected for *C. puteana* using western blotting, compared to at least 20 proteins visualised after SDS-PAGE and silver staining. The antigen profiles are therefore much simpler to interpret than the protein profiles. These simpler profiles allow easier recognition of *C. puteana* in wood thus facilitating identification. In addition, the streaking effect of extracts of highly degraded blocks evident with SDS-PAGE analysis, is absent in the western blotting system facilitating the interpretation of results for degraded blocks of all weight losses. Further, the western blotting technique is more sensitive, exhibiting the ability to detect low levels of *C. puteana* in decayed wood blocks, levels not detectable by SDS-PAGE.

Molecular profiles of *C. puteana* in a variety of situations are similar, therefore it is possible to use SDS-PAGE and western blotting for identification purposes. The consistent nature of *C. puteana* contrasts with other organisms, notably *S. lacrymans*. The identification of *S. lacrymans* in wood is possible (Palfreyman *et al.*, 1991a), but a greater variation in the profiles of extracts of the standard preparation of *S. lacrymans* and wood grown cultures is evident. The consistent nature of *C. puteana* in

comparison to the variation of *S. lacrymans* will now be discussed in more detail and with reference to Figure 5.17.

The analysis of *C. puteana* in chapter 4 indicated that the protein and antigen profiles of the GT, intermediate and aged regions of *C. puteana* mycelium shared common bands with each other, with the standard FPRL 11E and with the profiles of extracts of infected wood blocks (5.2.2.). The protein profile of the GT extract however exhibited a greater percentage similarity index value to the profiles of the extracts of infected wood samples (Table 5.5.), implying the possible presence of a growth phase molecular profile for *C. puteana*. The recognition of such a profile for *S. lacrymans* is evident and was easily recognised, due to the close resemblance of protein profiles of extracts of low percentage weight loss blocks (likely to contain mostly GT material) to the profile of the GT region of *S. lacrymans* (Vigrow, 1992).

To further investigate the possible presence of a growth phase molecular profile for *C. puteana*, western blotting analysis of the GT, WM and wood extracts of *C. puteana*, using an antiserum raised against growing tip material was carried out. The GT antiserum analysis showed the presence of sharp antigenic bands of similar molecular weights to fuzzy bands obtained using the WM antiserum. These sharp bands indicate the detection of immunogenic proteins which are specific to the GT region. That sharp bands are also detected in the WM extract using the GT antiserum is explained by the inclusion of the GT region in the initial preparation of the WM extract (2.3.1.). These antigenic bands cannot be directly related to proteins exhibited by SDS-PAGE and this suggests that they are highly antigenic.



KEY

CP	- *C. puteana*
SL	- *S. lacrymans*
*	- Antigenic representation
SDS-PAGE profile	- Protein band representation
#	- Low percentage weight loss blocks

Figure 5.17. Nature of variation of protein/antigens profiles of *C. puteana* cf. *S. lacrymans*.

These results indicate that both *C. puteana* and *S. lacrymans* have growth phase molecular profiles. There are however differences in the characteristics of these profiles. For *C. puteana*, the GT and WM extracts exhibit sharp protein bands when analysed by SDS-PAGE, fuzzy antigenic bands when analysed by western blotting using the WM antiserum and sharp antigenic bands when examined using the GT antiserum. By contrast, for *S. lacrymans*, the distinction between fuzzy and sharp, GT and WM antigenic bands was more easily detected since only one antiserum, raised against WM immunogen, was required to distinguish them.

A possible explanation for the contrasting results between *C. puteana* and *S. lacrymans* may relate to the position of the antigenic bands detected. Since the sharp antigen bands of *C. puteana* have similar molecular weights to the fuzzy antigen bands, the sharp GT antigen bands may be present within the fuzzy region, but are obscured due to the high antigenicity of the fuzzy antigens in reaction with the WM antiserum. By contast, the sharp GT antigen bands of *S. lacrymans* are of different molecular weights to the fuzzy WM antigen bands detected using the WM *Serpula* antiserum and are therefore more easily recognised. Further, the GT antigens of *C. puteana* are highly immunogenic since, despite the presence of WM antigens within the GT region, only GT antigens are detected.

The contrasting results of the two organisms may also be due to the accuracy of determining the GT region. It is possible that the GT region of *C. puteana* is smaller than that of *S. lacrymans* and that if a narrower band of *C.*

puteana mycelia had been used in western blotting, the WM antiserum of this organism might have detected just the sharp antigenic band. Nevertheless, evidence for the presence of growth phase antigens for *C. puteana* is provided by this data and further research of these antigens may reveal information on the decay status of the organism in wood.

Since drying is a major method for the prevention of growth of *C. puteana*, an analysis of specific changes in protein/antigen profiles of extracts of dried infected wood blocks was undertaken. If changes were found then the possibility of monitoring the drying process and thus the decrease in the viability of *C. puteana* could be addressed. However the data shown revealed that desiccation had no affect on the molecular nature of *C. puteana*. Therefore regardless of the moisture content of infected wood, the protein and antigen profiles of *C. puteana* remained constant (for at least 12 weeks) preventing specific effects of the drying process to be investigated, and highlighting the tolerance of the organism to environmental change.

Of the organisms that have been studied to date those which display variability in their molecular profiles in experimental systems eg. *S. lacrymans*, *C. versicolor* and *L. lepideus* (Palfreyman *et al.*, 1988a; Glancy, 1990; Palfreyman *et al.*, 1991a; Vigrow *et al.*, 1991a), are also sensitive to environmental changes. By contrast those which exhibit consistent profiles eg. *C. puteana* are not sensitive to the environment. The lack of sensitivity of *C. puteana* to environmental change is demonstrated by its apparent tolerance to high moisture levels and increased

temperatures (Cartwright and Findlay, 1958; Bech-Anderson, 1992). As a result of the lack of environmental sensitivity, *C. puteana* is used as a standard organism in the EN 113 testing of preservatives.

5.6.2. DETECTION OF *C. PUTEANA* IN WOOD

The complexity of SDS-PAGE profiles and the potential simplicity of immunological techniques indicate that the detection and identification of *C. puteana* in wood would be more easily undertaken using the latter techniques. Using western blotting the 3 immunological reagents developed showed the ability to detect *C. puteana* in wood at varying stages of decay and in addition there was no reaction with undecayed wood.

More appropriate detection methods than western blotting are EIA and dot immunoblotting, which are simple, provide a high throughput of samples, are quantitative and semi-quantitative respectively and can provide results rapidly. In this study EIA was used to detect *C. puteana* at various stages of decay with a variety of reagents. Antisera raised against WM and EP extracts of *C. puteana* allowed the detection of this organism in wood blocks using both WM and EP extracts of the blocks. Detection at all stages of decay was evident. In addition the reagents allowed the differentiation between various levels of decay by a corresponding increase in optical density with an increase in weight loss of infected blocks. Both antisera exhibited a similar ability to discriminate between different levels of decay, although the WM antisera produced the highest signals in the EIA test. Other wood decay organisms which can be detected by EIA include *P. placenta* (Jellison and

Goodell, 1988), *Ophiostoma* species (sap staining organisms; Breuil *et al.*, 1988a, b) and *H. annosum* (forest pathogen; Galbraith, PhD. thesis in preparation). Each of these organisms has been detected in laboratory decayed wood, and in each case, the immunological reagents utilised were antisera raised only against whole mycelial material and not against exoproteins.

The dot immunoblotting technique was also used to detect *C. puteana* in wood. However, detection by this method was unsuccessful due to the non-specific binding of the primary antisera to the wood, regardless of pre-absorption of the antisera with uninfected sawdust. This is in contrast to western blotting where no non-specific binding was found. The dot immunoblotting results suggest that the antibodies of the primary antiserum bind non-specifically through the Fc region of the antibody rather than the antigen binding site. Jellison and Goodell (1989) were faced with a similar non-specific binding problem in their EIA for the detection of *P. placenta* which could however be overcome by the dilution of the wood/fungal sample. In studies on *L. lepideus*, Palfreyman *et al.*, (1987) and Glancy *et al.*, (1989) using dot immunoblotting, successfully detected antigens of this organism in laboratory decayed wood blocks prior to appreciable weight loss and in pole stubs in the field. For these analyses, pre-absorption of antisera with uninfected wood overcame the non-specific binding problem encountered which related to the antigen binding site of the antibodies.

Additional problems with non-specific binding of reagents have been exhibited with *S. lacrymans* where the organism itself non-specifically binds reagents (Koch, Danish

Technological Institute; Palfreyman *et al.*, 1988a; Vigrow, 1992). Interestingly wood decay fungi and their corresponding antisera behave differently when tested in different immunological assays, such as, dot immunoblots and western blots. Both the wood/antisera and the organism/antisera non-specific binding exhibited by *C. puteana* (M^{c}Dowell *et al.*, 1992) and *S. lacrymans* (Palfreyman *et al.*, 1991a) in dot immunoblotting were not apparent in western blotting. This may be a reflection of the denaturing capacity of the reagents used in western blotting.

The dilemma of non-specific binding of immunological assay reagents is widespread. For example, methods for the detection of fungal disease of plant tissue have encountered this problem (Aguelon and Dunez, 1984; O'Connell *et al.*, 1986; Dewey and Brasier, 1988). Although this phenomenon for *C. puteana* and other fungi may be difficult to resolve (Aguelon and Dunez, 1984), it may be overcome by the production of higher affinity antiserum and/or, as described in this thesis, by the development of monoclonal antibodies.

Despite non-specific binding problems, immunological reagents can be used successfully for the analysis of fungal organisms involved in timber decay. For example, Kim (1991) described the immuno-localisation of extracellular metabolites from *T. palustris* in the wood cell, by transmission electron microscopy, using an antiserum raised against the culture filtrate of the organism. The detection of the organism in the wood cell was apparent which provided information on the penetrability of metabolites which may be essential for initial decay. In addition, the

analysis of *L. lepideus* in wood blocks of various weight losses using immunofluorescence has been described, which although using highly cross-reacting antisera, exhibited little non-specific binding of reagents (Glancy, 1990). It is difficult to be definitive about the likelihood of non-specific binding when utilising fungal/antibody based systems, probably due to the complexity of the immunogen/antigen. Therefore any new systems must be analysed carefully for potential non-specific binding by the inclusion of appropriate controls.

In addition to high backgrounds in assays due to non-specific binding of assay components, high cross-reactivity may also be a problem. The polyclonal antisera described for *C. puteana* are highly cross-reactive, as are antisera to other wood decay fungi, viz., *L. lepideus*, *C. versicolor*, *S. lacrymans*, *H. annosum*, *P. placenta*, *L. edodes* and *T. palustris* (Palfreyman *et al.*, 1988a; Vigrow *et al.*, 1991a; Kim *et al.*, 1989, 1991). The immunological reagents, are of limited use as probes for the specific identification and detection of wood decay organisms in simple systems. There are however certain advantages in having relatively non-specific antisera. For example, in timber such as some of those on the *Unicorn* and *Discovery* (see chapter 6), the specific identification of a particular organism may be less important than the detection of colonising wood rotting basidiomycetes. In certain circumstances, identification of specific organisms may be required, e.g. the treatments to eradicate the wet rot fungus *C. puteana* and the dry rot fungus *S. lacrymans* are different, and therefore fungal identification is necessary before treatment.

The potential for the development of more specific probes for *C. puteana* is highlighted in the discovery of unique exoantigens of this organism in comparison to other members of the genus (chapter 3). Unique exoantigens have been found for a range of other fungi e.g. *Histoplasma capsulatum, Coccidioides immitis* and *Blastomyces dermititidis;* these exoantigens being used in the immunodiagnosis of disease (Kaufman and Standard, 1987). An exoantigen extract was chosen in this project as an immunogen for the production of monoclonal antibodies to *C. puteana*, based on the use of this extract in other systems (Kaufman and Standard, 1987; Dewey *et al.*, 1989 and MacDonald *et al.*, 1989). In addition, extracts of exoantigens are easy to prepare and the exoantigens themselves are highly immunogenic since no adjuvants are required to produce an immune reaction.

The production of immunological reagents for the specific detection of *C. puteana*, viz., monoclonal antibodies, is described in this chapter, although only limited cross-reactivity studies were undertaken. The development of a specific probe for *C. puteana* was not possible, though a monoclonal antibody which reacted with the organisms within the genus *Coniophora* tested was produced. This probe allowed the detection of *C. puteana* antigens at all weight losses and most importantly at minimal levels. In addition, the monoclonal antibody possessed the ability to detect *C. puteana* in two different extracts ie. WM and EP, the former showing the largest optical density values in an EIA system. As well as detecting *C. puteana* in infected wood the specific MAbs produced during this project overcame the problem of non-specific binding to uninfected wood. Similar non-specific binding problems found in other systems have

also been solved by the production of monoclonal antibodies (MacDonald *et al.*, 1989; Dewey *et al.*, 1989; Jellison and Goodell, 1986; Galbraith, PhD. thesis in preparation).

Due to the use of monoclonal antibodies, many of the problems associated with polyclonal antisera have been overcome and a variety of specific probes to wood decay fungi have now been produced. For example, the production of MAbs to extracellular metabolites of *P. placenta* (Jellison and Goodell, 1986) and to manganese (II) peroxidase in wood degraded by the white rot organism *Phanerochaete chrysosporium* (Daniel *et al.*, 1991) has allowed the detection of these organisms in wood and has provided an insight into their mechanisms of decay. The detection of the forest pathogen *H. annosum* in laboratory decayed wood and in the standing tree, by MAbs, has allowed the possibility of the development of a monitoring system for the infection of trees by this organism, (Galbraith, PhD. thesis in preparation). Similarly the detection of *S. lacrymans* in laboratory decayed wood using MAb has been achieved, allowing the early detection of the organism in wood and providing the basis for an on-site system for the detection of the organism (Glancy, personal communication).

CHAPTER SIX

APPLICATION OF SYSTEMS DEVELOPED TO MARITIME ARTIFACTS

6.1. INTRODUCTION

Studies of *C. puteana* proteins and antigens described in chapter 3 allowed the establishment of a preliminary identification system for *C. puteana* and related organisms grown on standard media. The molecular and immunological characterisation of *C. puteana* proteins and antigens in chapter 4 provided evidence of the conserved nature of *C. puteana* WM proteins and antigens which allowed the production of simple profiles for identification studies, the information from which was utilised in chapter 5, for the development of a preliminary detection system for organisms in wood.

The current study, describes the application of the identification systems for *C. puteana*, to other wet rot fungi colonising the timbers of the frigate *Unicorn* and RRS *Discovery*. In addition the feasibility of the specific detection system described in chapter 5 for members of the genus *Coniophora* in wood, was assessed, by its application to timbers in diagnosed wet rot locations on the *Unicorn*.

6.2. IDENTIFICATION OF ORGANISMS FROM THE FRIGATE *UNICORN* AND RRS *DISCOVERY*

The identification of organisms from the historic ships under study, can be divided into 3 phases. The first was the identification of organisms isolated from initial samples removed from the ships in 1989, which included fruiting bodies, infected wood, and mycelial and strand samples; the second was the analysis of strand and mycelial material removed from the *Unicorn* in 1990 subsequent to the discovery of wet rot which was consistent with an infection

of *C. puteana*; the third was the analysis in 1991 of organisms isolated from wooden core samples removed from the area of the *Unicorn* attacked by *C. puteana*.

6.2.1. IDENTIFICATION OF INITIAL ISOLATES

All samples from the maritime artifacts were described morphologically on removal from the timber and again once cultures obtained from such samples were established. Pure mycelial cultures exhibiting masses of aerial mycelia, growth over agar medium in a petri-dish within 2-3 days, microscopic presence of fruiting structures and the absence of clamp connections, were characteristic of mould growth and were thus discarded. Other isolates which did not exhibit the features of moulds and which possessed clamp connections (basidiomycetes), were utilised in further studies and are detailed in Table 6.1.

Studies of standard texts on wood decay fungi (Cartwright and Findlay, 1958; Bravery, 1984 and Coggins, 1984), provided useful information towards the identification of some cultures described in Table 6.1. From this information it was possible to positively identify one of the organisms, D3, as *L. sulphureus*. This identification was accomplished due to the characteristic yellow fruiting body of *L. sulphureus* which produces distinctive salmon pink mycelia in culture. In addition U3, U5 and U6 may be *P. placenta* due to the presence of yellow-cream fruiting bodies (slightly tinged with pink) which contain minute pores, features of such structures of this organism. Furthermore, characteristic yellow-white mycelia were evident.

TABLE 6.1.

MORPHOLOGICAL DESCRIPTIONS OF SAMPLES REMOVED FROM THE *UNICORN* (U) AND *DISCOVERY* (D)

KEY

* - Isolation date, 13/4/89.
\+ - Isolation date, 18/9/89.
1. - Description of sample removed from ship.
2. - Description of mycelia isolated from sample.
FB - Fruiting body.
CC - Clamp connections.

SAMPLE AND LOCATION	DESCRIPTION	COMMENT
PART A, *UNICORN* SAMPLES (U1-U6, Upper deck; U9-U20, Forward hold)		
U1, Beam above Forecastle at position 14. 350mm from starboard side.*	1. Thick light brown coloured FB. 2. In culture mycelia are white, short and felt-like.	1. FB similar to *Donkioporia expansa*. 2. Culture not similar to this organism, since mycelia are not red.
U2, Beam above Forecastle at position 14. 350mm from starboard side.*	1. Thick light brown coloured FB. 2. In culture mycelia are white, short and felt-like.	1. FB similar to *Donkioporia expansa*. 2. Culture not similar to this organism since mycelia are not red.
U3, 8th beam from fore end. Front side of beam 1000mm from starboard.*	1. Yellow-cream FB with minute pores and slight pink tinge. 2. Yellow-white mycelia in culture.	1. FB similar to *Poria placenta*. 2. Culture similar to mycelia of *P. placenta*.
U4, Perimeter of "down-pipe" for rain-water, 95mm from starboard at fore of ship.*	1. Brown-red FB. 2. White mycelia produced.	1. FB indicates presence of a basidiomycete.
U5, Fore of first beam on rounded section of ship.*	1. 80mm length cream-brown FB. 2. In culture 2 mycelial types; white and dense; sparse and fine.	1. FB similar to *P. placenta*.
U6, Fore of first beam on rounded section of ship.*	1. 120mm length cream-brown, thick, tough FB. 2. In culture 2 mycelial types; white and dense; sparse and fine.	1. FB similar to *P. placenta*

SAMPLE AND LOCATION	DESCRIPTION	COMMENT
U9, Port in forward hold of hull, beside grate.+	1. Decayed wood with white mycelial spots. 2. Mycelia white and fluffy in culture.	1. Possibly a basidiomycete. 2. Confirmed basidiomycete mycelia, tested by CC.
U12, Starboard side of hull. One frame to the right of beer barrels, between 2nd and 3rd thick stuff.+	1. Yellow mycelia on decayed wood. 2. In culture 2 mycelial types; white and dense; sparse and fine	1. Possibly a basidiomycete. 2. Confirmed basidiomycete mycelia, tested by CC.
U13, Port side of hull, beside beer barrels.+	1. Yellow-cream mycelia on decayed wood. 2. Mycelia yellow-cream in culture.	1. Possibly a basidiomycete. 2. Confirmed basidiomycete mycelia, tested by CC.
U17, Outer frame of ship, from starboard side on water line down from second window on bow.+	1. 250mm length of brittle wood, some parts obviously decayed, wood not wet. 2. In culture 2 mycelial types; white and dense; sparse and fine.	1. Possibly a basidiomycete. 2. Confirmed basidiomycete mycelia, tested by CC.
U20, In the bilge water, next to keelson on the port side in the forward hold.+	1. Slimy brown hyphal strand floating on water. 2. Yellow-cream woolly mycelia in culture.	1. Possibly *C. puteana* strand. 2. Mycelia produced are more similar to *C. marmorata*.

SAMPLE AND LOCATION	DESCRIPTION	COMMENT
PART B, *DISCOVERY* SAMPLES		
DIc, Timber removed from port side of *Discovery*.+	1. Decayed wood from D timber. 2. White mycelia in culture.	1. Possibly a basidiomycete. 2. Confirmed basidiomycete mycelia, tested by CC.
D3f, Timber removed from port side of *Discovery*.+	1. Decayed wood from D timber. 2. White mycelia in culture.	1. Possibly a basidiomycete. 2. Confirmed basidiomycete mycelia, tested by CC.
D3, Oak panel in cabin on *Discovery*.+	1. Distinctive sulphur yellow FB. 2. Salmon pink mycelia in culture.	1. FB similar to *Laetiporus sulphreus*. 2.Characteristic pink mycelia of *L. sulphureus* present.

The preliminary identification of the strand sample U20 as a member of the *Coniophora* genus was possible. It was thick, brown and in places flattened onto the wood surface characteristic of *C. puteana* (Ginns, 1982). However, the mycelia produced from this strand sample was not similar to the standard culture of *C. puteana* FPRL 11E, since it did not possess floccose mycelia with an even advancing defined margin. In addition, the older mycelia of the agar culture of U20 did not reflect the appearance of older mycelia of *C. puteana* FPRL 11E i.e. it did not produce appressed, brown hyphae. Instead the mycelia of sample U20 was more similar to that of *C. marmorata*, exhibiting the characteristic white-brown mycelia which is densely downy to sparsely woolly with an uneven margin, in which faster growing fan shaped sections are evident (Figure 6.1.). No other common features of the organisms detailed in Table 6.1., with fungi described in the reference works used in this analysis, were evident.

Table 6.2. shows the growth rates of all isolated basidiomycetes from Part A and B of Table 6.1. The growth rates can be divided into 3 groups viz., Group I, 7-10mm/day; Group III, <4mm/day and an intermediate group, Group II, containing only one isolate. Similarities within each group can sometimes indicate a relationship of isolates to each other, for example, U52 and U61 with growth rates of 8.9mm/day and D3f, DIc with growth rates of 3.7mm/day. Both pairs of organisms were subsequently shown to be of the same type i.e. U52, U61 - *P. placenta* and D3f, DIc - *H. puberum*; (see later). However growth rates cannot

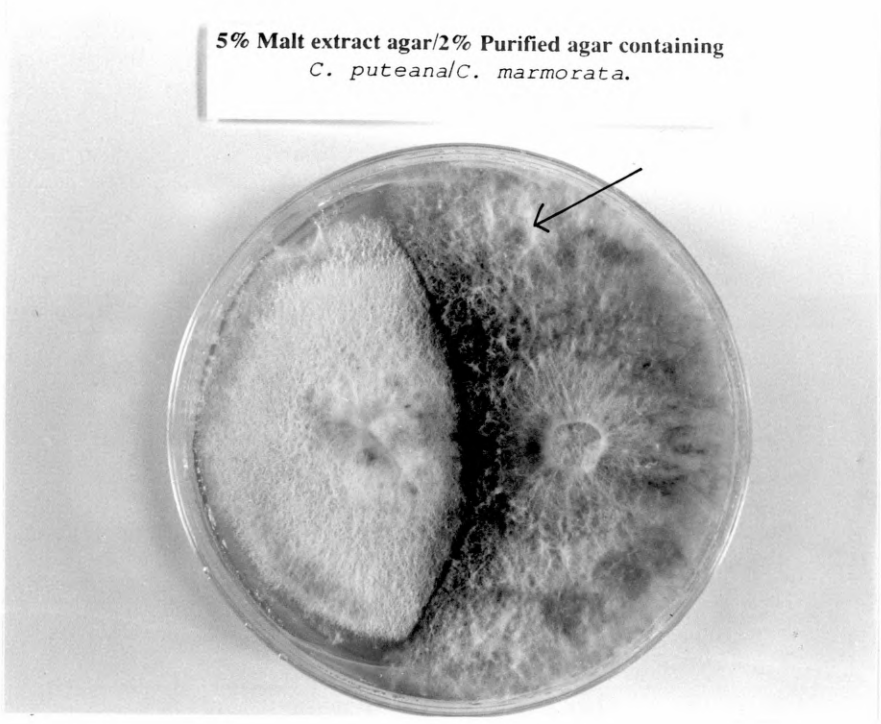


FIGURE 6.1. Comparison of laboratory cultured standard mycelial samples of *C. puteana* and *C.marmorata*. The cream-white floccose mycelia possessing an even hyphal front is evident for *C. puteana*, whilst white-brown mycelia which is densely downy to sparely woolly is evident for *C. marmorata*. The most distinguishing features of *C. marmorata* is the presence of an uneven margin and also the presence of fast growing fan shaped sections of mycelia (⟶).

ISOLATES	RADIAL GROWTH RATES (mm/day)
GROUP I	
U20	9.6
D3	7.8
U4	7.3
U52	8.9
U61	8.9
U12b	7.8
U17	7.8
U17b	7.8
GROUP II	
U3	5.3
GROUP III	
U9	2.3
U51	1.8
U62	1.3
U12	2.2
D3f	3.7
DIc	3.7
U13	2.5
U2	1.0
U1	1.4

TABLE 6.2.

GROWTH RATES OF ISOLATES FROM THE *UNICORN* AND *DISCOVERY*

be used alone and require to be supplemented with some other method for identification, for example, morphological comparison and microscopic analysis of mycelia, or molecular analysis of the macromolecules within the hyphae, using techniques such as SDS-PAGE.

The use of SDS-PAGE in the identification process for all organisms in this section, was by initial reference to *C. puteana* FPRL 11E (Figure 6.2.). Although isolate U20 (track 6) is the only organism which exhibits a similar protein profile to that of FPRL 11E, similarity between the profiles of some isolates was observed viz., D3f and DIc (tracks 2, 3). The profiles of the isolates were compared to the profiles of other basidiomycetes (Figures 6.3., 6.4. and 6.5.). Figure 6.3. highlights the differentiation of some isolates (tracks 9-12) from each other and from a range of basidiomycetes, whilst Figure 6.4. indicates the identification of isolates U61, U4 and U52 (tracks 2, 4 and 6 respectively) as *P. placenta* (tracks 1, 3). Figure 6.5. confirms the identity of isolate D3 (track 12) as *L. sulphureus* (tracks 11, 13, 15, 17) and substantiates previous evidence of the identity of isolate U20 (track 9) as a member of the *Coniophora* genus (tracks 8, 10).

Having compared all isolates (total of 18) to FPRL 11E and to those relevant organisms available at DIT, 5 isolates gave protein profiles which resembled those of a range of organisms once classified into the *Poria* genus i.e. *P. placenta*, *P. incrassata*, *F. vaillantii* and *A. xantha* (Table 6.3.a and b). These organisms were once classified into the same genus, viz., *Poria* (Cartwright and Findlay, 1958). Eight isolates remained unidentified after this analysis. Five of these isolates were analysed by morphological and

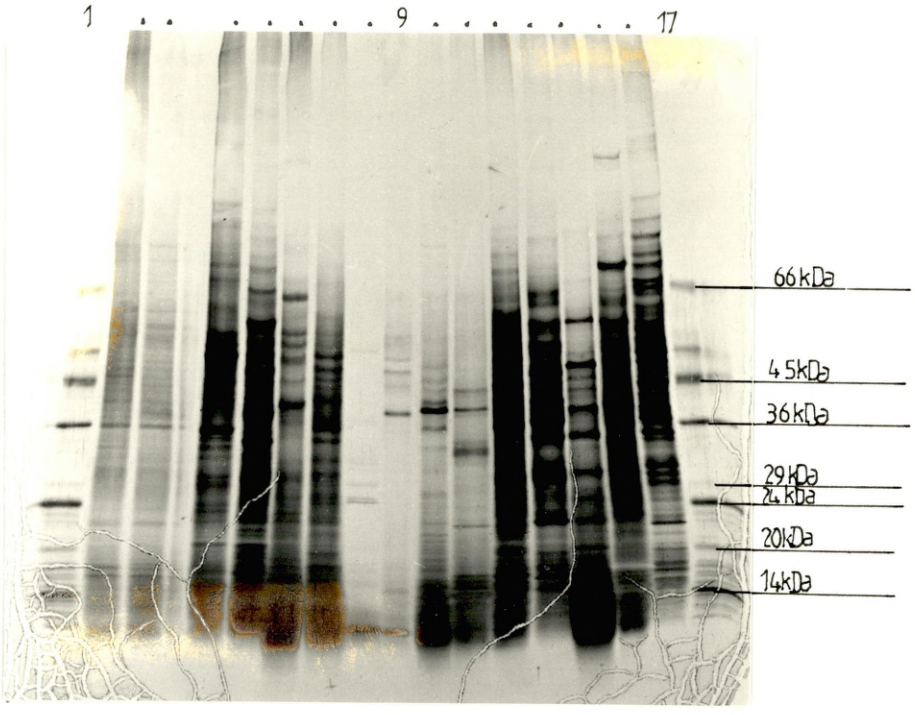


FIGURE 6.2. SDS-PAGE identification of isolated fungal organisms from the *Unicorn* and *Discovery*. Tracks represent: 1, 17 - Standard molecular weight markers (MWM); 2 - D3f; 3 - DIc; 4 - U17b; 5 - U17; 6 - U20; 7 - U12; 8 - U12b; 9 - U9; 10 - *C. puteana* FPRL 11E; 11 - *S. lacrymans* FPRL 12C; 12 - *A. xantha* FPRL 62F; 13 - *F. vaillantii* FPRL 14H; 14 - *L. lepideus* FPRL 7F; 15 - *P. incrassata* FPRL 71; 16 - *D. quercina* FPRL 38. In this and all subsequent figures the molecular weight markers were of the following sizes, 14,200, 20,100, 24,000, 29,000, 36,000, 45,000 and 66,000 Daltons.

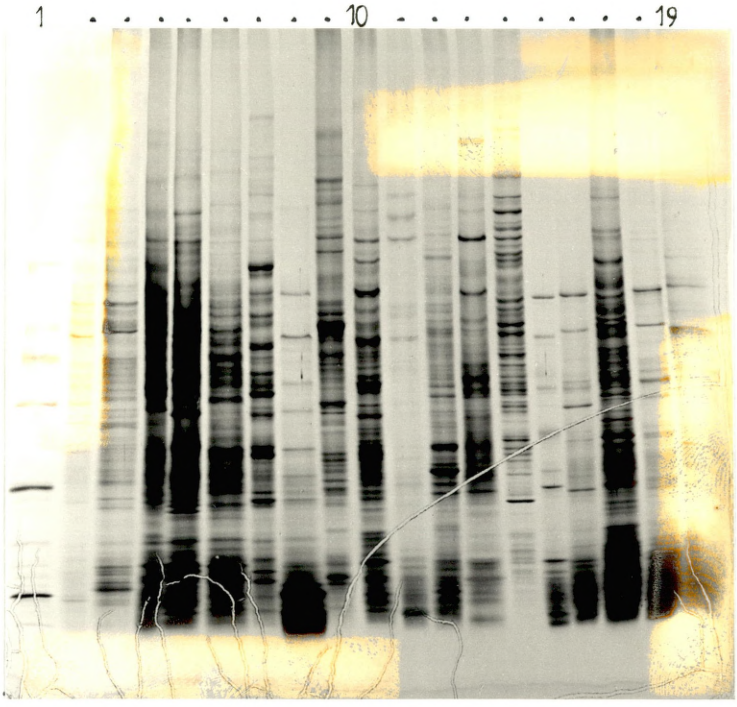


FIGURE 6.3. SDS-PAGE identification of isolated fungal organisms from the *Unicorn* and *Discovery*. Tracks represent: 1 - MWM (Figure 6.2.) ; 2 - *C. puteana* FPRL 11E; 3 - *S. lacrymans* FPRL 12C; 4 - *P. placenta* FPRL 280; 5 - *P. ostreatus* FPRL 40A; 6 - *A. xantha* FPRL 62F; 7 - *F. vaillantii* FPRL 14H; 8 - *L. lepideus* FPRL 7F; 9 - U13; 10 - U17b; 11 - U1; 12 - U12b; 13 - *P. incrassata* FPRL 71; 14 - *D. quercina* FPRL 38; 15 - *G. trabeum BAM 109; 16 - G. sepiarium FPRL 10D; 17 - P. gigantae* FPRL 175B; 18 - *S. pinastri* FPRL 141B; 19 - *L. sulphureus* FPRL 29.

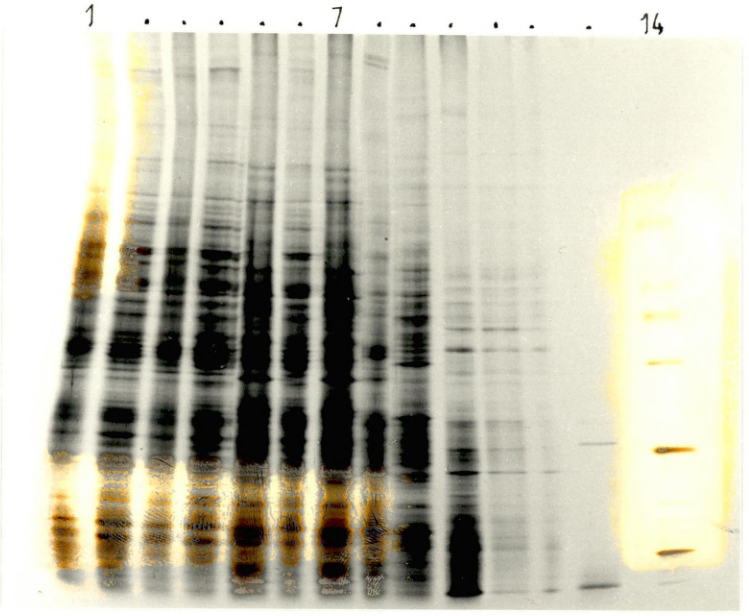


FIGURE 6.4. SDS-PAGE identification of isolated fungal organisms from the *Unicorn* and *Discovery*. Tracks represent: 1, 3 - *P. placenta* FPRL 280; 2 - U61; 4 - U4; 5, 7 - *P. ostreatus* FPRL 40A; 6 - U52; 8 - U3; 9 - *A. xantha* FPRL 62F; 10 - U51; 11 - U62; 12 - U9; 13 - U12; 14 - MWM (Figure 6.2.)

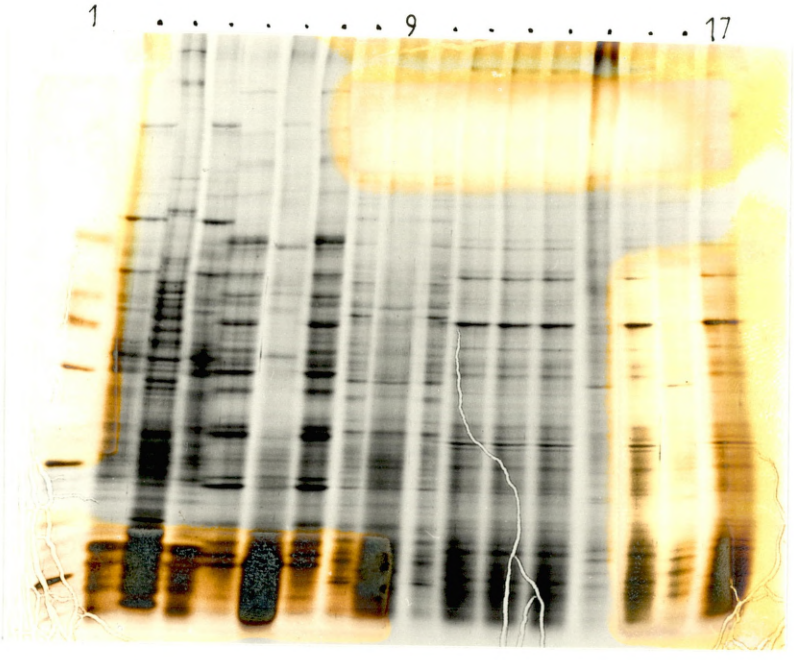


FIGURE 6.5. SDS-PAGE identification of isolated fungal organisms from the *Unicorn* and *Discovery*. Tracks represent: 1 - MWM (Figure 6.2.); 2, 4 - *P. incrassata* FPRL 71; 3 - U2; 5, 7 - *F. vaillantii* FPRL 14H; 6 - U17; 8, 10 - *C. puteana* FPRL 11E; 9 - U20; 11, 13, 15, 17 - *L. sulphureus* FPRL 29; 12 - D3; 14 - D3f; 16 - DIc.

SAMPLES	ISOLATES	SIMILARITY OF ISOLATES AFTER SDS-PAGE	ORGANISM IDENTITY AT DIT
U1	U1 (1)		-
U2	U2 (2)	x	-
U3	U3		FV/PP
U4	U4	*	PP
U5	U51	+	FV/PP/AX/PI
	U52	*	PP
U6	U61	*	PP
	U62	+	FV/PP/AX/PI
U9	U9	+	FV/PP/AX/PI
U12	U12	+	FV/PP/AX/PI
	U12b (3)		-
U13	U13 (4)	x	-
U17	U17 (5)		-
	U17b (6)		-
U20	U20		CM
DIc	DIc (7)	#	-
D3f	D3f (8)	#	-
D3	D3		LS

14 samples produced 18 isolates, 10 of which were identified to varying degrees, 8 isolates (denoted 1-8) remain unidentified.

N.B. See Table 6.3.b for abbreviations of organism names.

TABLE 6.3.a

ANALYSIS OF SAMPLES FOR IDENTIFICATION USING SDS-PAGE

ABBREVIATIONS	ORGANISM NAMES
CP	*C. puteana*
CM	*C. marmorata*
LS	*L. sulphureus*
PP	*P. placenta*
FV	*F. vaillantii*
AX	*A. xantha*
PI	*P. incrassata*
PIg	*P. igniarius*
VLe	*V. lecanii*
HP	*H. puberum*
VL	*V. lamellicola*

TABLE 6.3.b

ABBREVIATIONS FOR ORGANISM NAMES USED IN TABLE 6.3.a AND SUBSEQUENT TABLES

ISOLATE	ORGANISM
DIc	*Hyphoderma puberum* CBS 464.86
U1	*Verticillium* cf. *lamellicola* CBS 912.70A
U2	*Verticillium lecanii* CBS 546.81
U13	*Verticillium lecanii* CBS 546.81
U17b	*Phellinus* cf. *igniarus* CBS 349.74

TABLE 6.4.

CONVENTIONAL IDENTITY OF ORGANISMS FROM CBS BAARN

microscopic characteristics at CBS Baarn, and their identity shown in Table 6.4. The reference fungi used to identify these isolates were made available to Dundee Institute of Technology and subsequently analysed using SDS-PAGE (Figure 6.6.). The visual analysis of the results confirmed that the identity of 3 of the isolates viz., DIc (track 3) as *Hyphoderma puberum*, and U2 and U13 (track 9) as *Verticillium lecanii*. By contrast, the profile of U17b (track 12; *Phellinus igniarius*; CBS Baarn), was more similar to the profile of the organism *V. lecanii* (track 8, 10) than that of *P. igniarius*. Although this casts some doubt on the identity of U17b, the two organisms *Phellinus* and *Verticillium* are so different that it is possible that inaccurate labelling may have caused some confusion; further analysis of these organisms is required. The visual analysis of isolate U1 appeared to confirm organism identity but the analysis was difficult due to the low concentration of protein in the sample. A summary of the possible identity of these organisms is shown in Table 6.5.

Numerical analysis of all the isolates is shown in Table 6.6. Visual identification of isolates U20, D3, U4, U52, U61, D3f, DIc, U13 and U2 was confirmed by their high similarity indices. It was not possible using numerical analysis to confirm the identity of U3, U9, U51, U62 and U12, and thus further analysis of a wider range of related organisms using SDS-PAGE is required.

In addition, further analysis is required to positively identify isolates U17b and U1, due to the relatively low similarities between these organisms and their proposed reference organisms. Overall, the analysis provided information on the possible identity of 16 isolates, only 2 isolates remaining completely unknown.

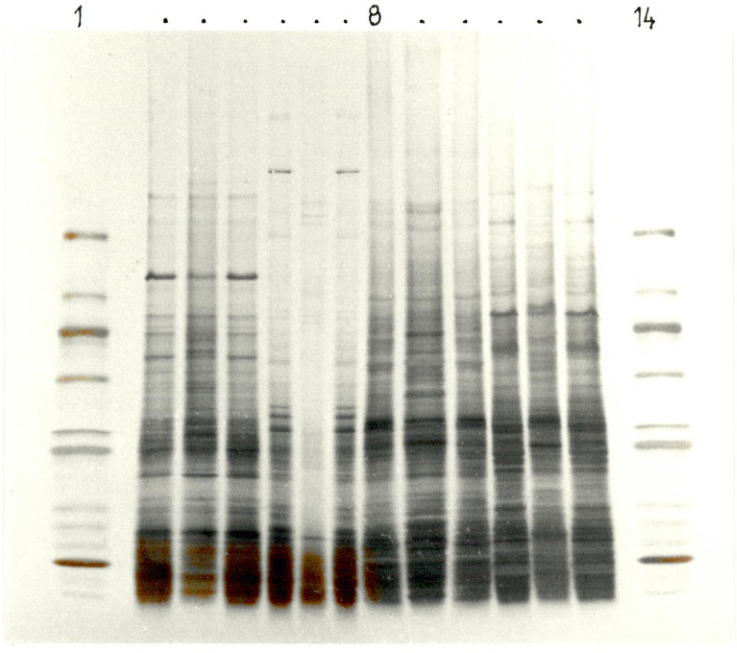


FIGURE 6.6. SDS-PAGE analysis of fungal organisms from the *Unicorn* and *Discovery* identified conventionally at CBS Baarn. Tracks represent: 1, 14 - MWM (Figure 6.2.); 2, 4 - *H. puberum* CBS 464.86; 3 - DIc; 5, 7 - *V. lamellicola* CBS 912.70A; 6 - U1; 8, 10 - *V. lecanii* CBS 546.81; 9 - U2; 11, 13 - *P. igniarius* CBS 349.74; 12 - U17b.

SAMPLES	ISOLATES	SIMILARITY OF ISOLATES AFTER SDS-PAGE	ORGANISM IDENTITY FROM CBS BAARN
U1	U1	VL#	VL
U2	U2	Vle	VLe
U12	U12b*	-	-
U13	U13	VLe	VLe
U17	U17*	-	-
	U17b	VLe##	PIg
DIc	DIc	HP	HP
D3f	D3	HP	HP

7 samples produced 8 isolates, 6 of which were identified.

KEY
* - Unidentified organisms.
# - Difficult to analyse by SDS-PAGE.
- Showed more resemblance to VLe than to PIg.

N.B. See Table 6.3.b for abbreviations.

TABLE 6.5.

IDENTITY OF SAMPLES AFTER ANALYSIS AT CBS BAARNE

ISOLATE	PERCENTAGE SIMILARITY TO REFERENCE FUNGI
GROUP I	
U20	CM - 91%
D3	LS - 94%
U4	PP - 97%
U52	PP - 82%
U61	PP - 97%
U12b	Unidentified
U17	Unidentified
U17b	PIg - 50%
U17b	VLe - 67%
GROUP II	
U3	FV - 68% PP - 55%
GROUP II	
U9	FV - 50% PP - 50% AX - 56% PI - 68%
U51	FV - 50% PP - 50% AX - 56% PI - 68%
U62	FV - 50% PP - 50% AX - 56% PI - 68%
U12	FV - 50% PP - 50% AX - 56% PI - 68%
D3f	HP - 95%
DIc	HP - 95%
U13	VLe - 85%
U2	VLe - 85%
U1	VL - 50%

TABLE 6.6.

PERCENTAGE SIMILARITY INDEX FOR ISOLATES FROM THE *UNICORN* AND *DISCOVERY*

6.2.2. IDENTIFICATION OF FUNGI RESPONSIBLE FOR FUNGAL INFECTIONS EXHIBITING WET ROT ON THE *UNICORN*

Dark brown thick strand material was found growing on the timbers of the frigate *Unicorn* in the forward hold, along the keelson and thick-stuff (at the port side adjacent to the keelson) and also in the bilge water (Figure 6.7.). The strand material was accompanied by 2 large fans of actively growing mycelia which were white at the hyphal tips and cream/brown in the older parts of the colony which also exhibited brown drops of exuded liquid (Figure 6.8A). The very oldest part of the colony was extremely sparse and formed brown strands behind the mycelial fan (Figure 6.8B). Figures 6.9. and 6.10., also show outbreaks of the same organism at 2 additional locations within the forward hold. This fungal attack on the *Unicorn* was consistent with infection by *C. puteana*, due to the presence of thick brown strand material, cream-white mycelia and the characteristic longitudinally cracked wood (Figure 6.9.; Coggins, 1980).

6.2.2.1. ANALYSIS OF MICROBIALLY ISOLATED ORGANISMS

Strand and mycelial samples (total of 13), removed from the *Unicorn* were analysed morphologically as detailed in Table 6.7. Provisional identification of the strand material as *C. puteana* was possible. However some mycelial cultures produced from the strands were more similar in appearance to mycelial cultures of *C. marmorata* mycelia (Figure 6.1.).

It was impossible to obtain pure mycelial cultures of samples Uf, Uj and Ul due to severe contamination with mould growth, highlighting a problem associated with microbial isolation. From the remaining 10 pure mycelial

FIGURE 6.7. Brown strands of a basidiomycete organism growing in the bilge water on the *Unicorn*. The strands can be seen growing from the thick stuff (TS) to the keelson (K) and back to the thick stuff.

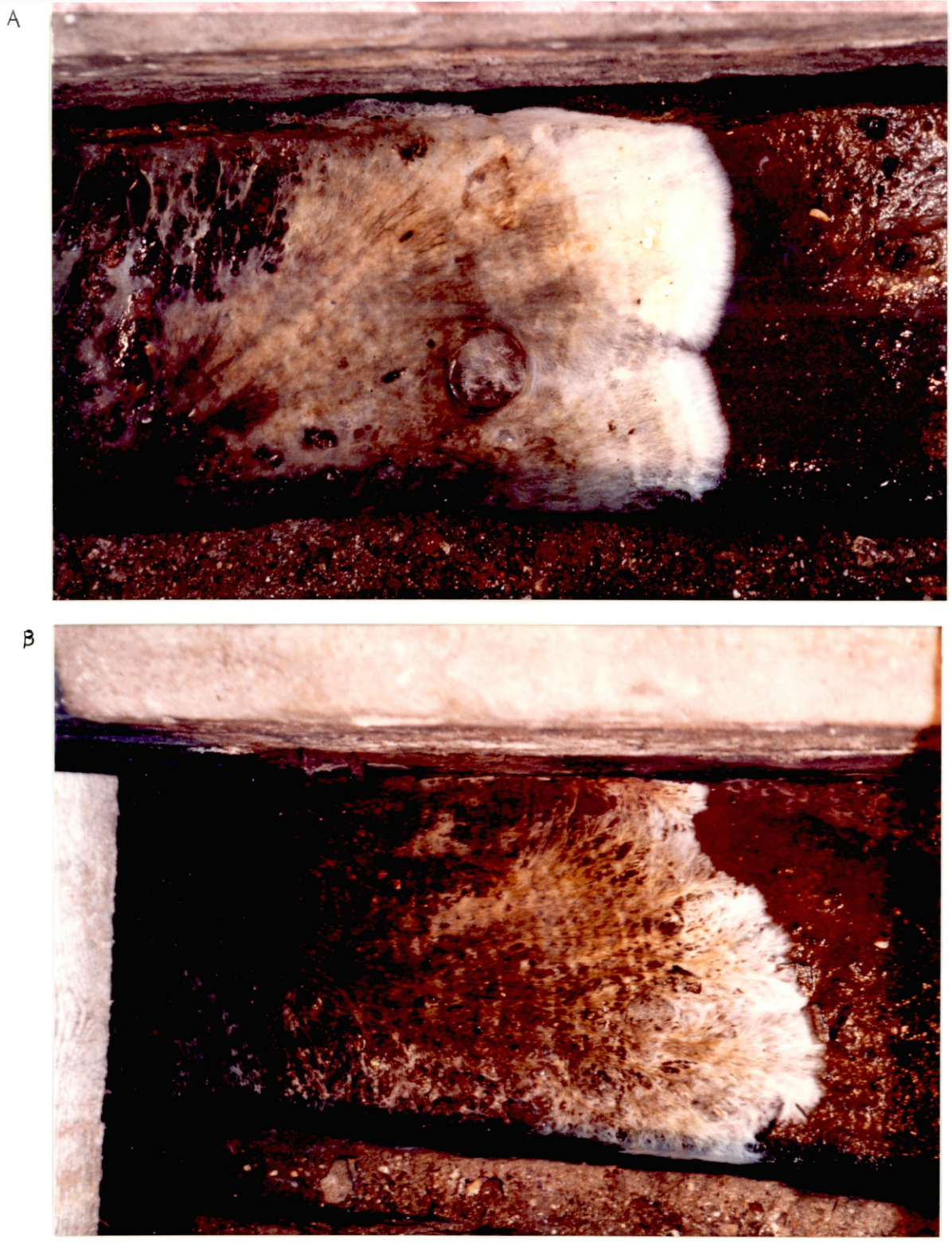


FIGURE 6.8. Mycelial fans of a fungal organism in the bilge water on the *Unicorn*. A - A dense mycelial mat is seen at the growing edge of the colony. B - Mycelial growth is preceded by brown hyphal strands.

FIGURE 6.9. Masses of hyphal strand growth over the frames of the *Unicorn* at the starboard side of the keelson. Actively growing mycelia can be seen at the edges of the hyphal strands.

FIGURE 6.10. Hyphal strand growth on thick stuff on the *Unicorn*, surrounded by mycelial fans.

TABLE 6.7.

MORPHOLOGICAL DESCRIPTIONS OF SAMPLES REMOVED FROM AREAS IN WHICH WET ROT WAS APPARENT

KEY - Samples Ua, Ub, Uc, Ud, Ue and Uh were located at the port side of the ship.
- Samples Uf and Ug were located at the starboard side of the ship.
- Samples Ui, Uj, Uk, Ul and Um were located at the port side of the keelson in the bilge water.
- All terms describing the location of samples are defined in Figures 2.4. and 2.5.

N.B. For a comparison between *C. puteana/C. marmorata* mycelia, see Figure 6.1.

SAMPLE AND LOCATION	DESCRIPTION	COMMENT
Ua, Thick stuff 2, stack 1.	1. Fine dry black-brown strands. 2. White-yellow woolly mycelia in culture.	1. Strands similar to *C. puteana*. 2. Mycelial growth similar to *C. marmorata*.
Ub, Thick stuff 1.	1. Fine black-brown strands. 2. White mycelia in culture.	1. Strands similar to *C. puteana*.
Uc, Between thick stuff 1 and 2, stack 3.	1. Fine dry black-brown strands. 2. White-yellow woolly mycelia in culture.	1. Strands similar to *C. puteana* 2. Mycelial growth similar to *C.marmorata*.
Ud, Between thick stuff 1 and 2, below stack 3.	1. Fine dry black-brown strands. 2. White mycelial spots produced with evidence of fructifications characteristic of mould growth.	1. Strands similar to *C. puteana*. 2. Strands not viable, producing moulds.
Ue, Between thick stuff 1 and 2, between stacks 3 and 4.	1. Fine dry brown strands. 2. White-yellow mycelia in culture.	1. Strands similar to *C. puteana*.
Uf, Between thick stuff 1 and 2, between stacks 3 and 4	1. Fine mycelia with crumbled wood. 2. White mycelia produced in culture, but contaminated with mould growth.	1. Possibly basidiomycete mycelia. 2. Mycelial growth due to moulds.
Ug, Between thick stuff 1 and 2, between stacks 3 and 4.	1. Fine mycelia with crumbled wood. 2. White mycelia produced in culture, but contaminated with mould growth.	1. Possibly basidiomycete mycelia. 2. Mycelial growth due to moulds.

SAMPLE AND LOCATION	DESCRIPTION	COMMENT
Uh, Thick stuff 2, stack 1.	1. Dried thick black-brown strands. 2. White-yellow woolly mycelia in culture.	1. Strands similar to *C. puteana*. 2. Mycelial growth similar to *C. marmorata*
Ui, Bow between stacks 3 and 4.	1. Thick wet black-brown strands. 2. White-yellow woolly mycelia in culture.	1. Strands similar to *C. puteana*. 2. Mycelial growth similar to *C. marmorata*.
Uj, Bow between stacks 3 and 4.	1. Thick black-brown strands. 2. White-yellow mycelia contaminated with mould growth.	1. Strands similar to *C. puteana*
Uk, Bow between stacks 3 and 4, under stack 3.	1. Thick black-brown strands. 2. Mycelia showing characteristics mould growth.	1. Strands similar to *C. puteana*.
Ul, Bow at stack 4.	1. Medium thick brown strands. 2. Mycelia contaminated with mould growth.	1. Strands similar to *C. puteana*.
Um, Bow between stacks 4 and 5.	1. Large fan of fluffy viable white-creamy mycelia. 2. Similar woolly-like mycelia in culture.	1. Mycelia similar to cultures of *C. marmorata*. 2. Mycelial growth similar to *C. marmorata*.

cultures, 7 possessed clamp connections and the remaining 3 possessed macro- and microscopic features characteristic of mould growth (samples Ud, Ug and Uk). This indicates the possibility that the strand samples Ud and Uk may have been non-viable structures.

Figure 6.11., shows the SDS-PAGE analysis of all mycelial cultures isolated from the samples detailed in Table 6.5., in comparison to the reference organism *C. puteana* FPRL 11E. Isolates Ua, Ub, Uc, Ue, Uh, Ui and Um (tracks 3, 4, 5, 7, 8, 9, 11) have profiles similar to *C. puteana* whereas isolates Ud, Ug and Uk (tracks 13, 14, 15) exhibit marked differences. This analysis is confirmed by the percentage similarity index shown in Table 6.8. (20%-33% for Ud, Ug and Uk; 50%-73% for the remaining isolates). Using the criteria established in chapter 3, isolates Ua, Ub, Uc, Ue, Uh, Ui and Um, can be identified as members of the *Coniophora* genus but their identification as *C. puteana* is not confirmed.

Table 6.8. shows the substantial similarity of the isolates to a representative isolate Ub and suggests that the isolates are more related to Ub and to one-another (mean value 84%) with the possible exception of Ui, than to *C. puteana* (mean value 60%). The morphological descriptions of the mycelial cultures in Table 6.7., suggested that the isolates were more similar to cultures of *C. marmorata* than to *C. puteana*. To confirm this a map of the protein pattern of these organisms in comparison to isolate Ub was constructed and similarity values calculated (Figure 6.12.). Isolate Ub clearly shares a greater similarity to *C. marmorata* than to *C. puteana* (93% cf. 57%). Since all

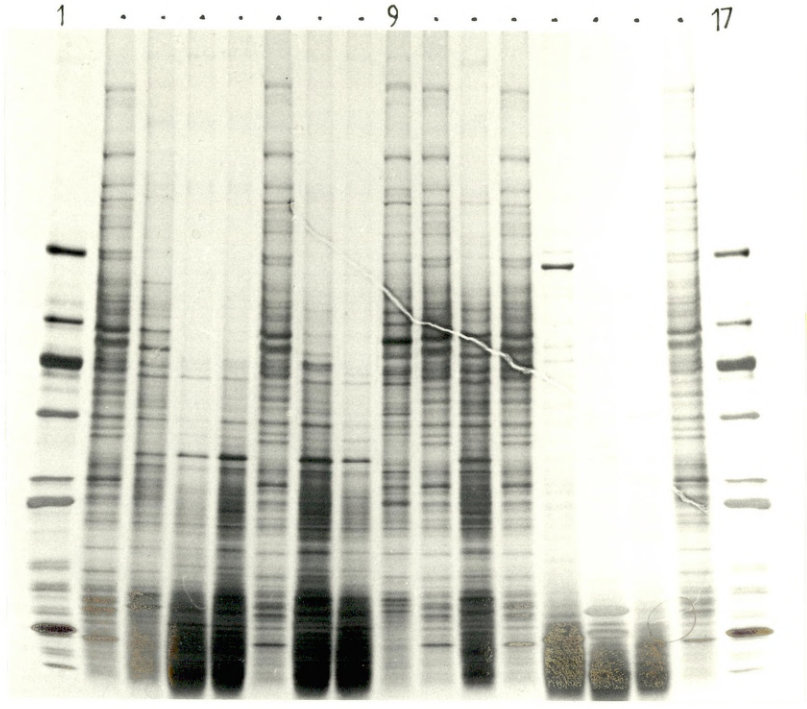


FIGURE 6.11. SDS-PAGE analysis of isolated mycelia from samples removed from the *Unicorn*. Tracks represent: 1, 17 - MWM (Figure 6.2.); 2, 6, 10, 12, 16 - *C. puteana* FPRL 11E; 3, 4, 5 - isolates Ua, Ub, Uc; 7, 8, 9 - isolates Ue, Uh, Ui; 11 - isolate Um; 13, 14, 15 - isolates Ud, Ug, Uk.

TEST	CP	Ua	Ub	Uc	Ue	Uh	Ui	Um	Ud	Ug	Uk
REF											
CP	100	58	50	55	67	53	73	62	33	24	20
Ub	68	83	100	88	95	88	68	80	43	35	33

TABLE 6.8.

PERCENTAGE SIMILARITY INDEX FOR MYCELIAL ISOLATES OF SAMPLES FROM THE *UNICORN*

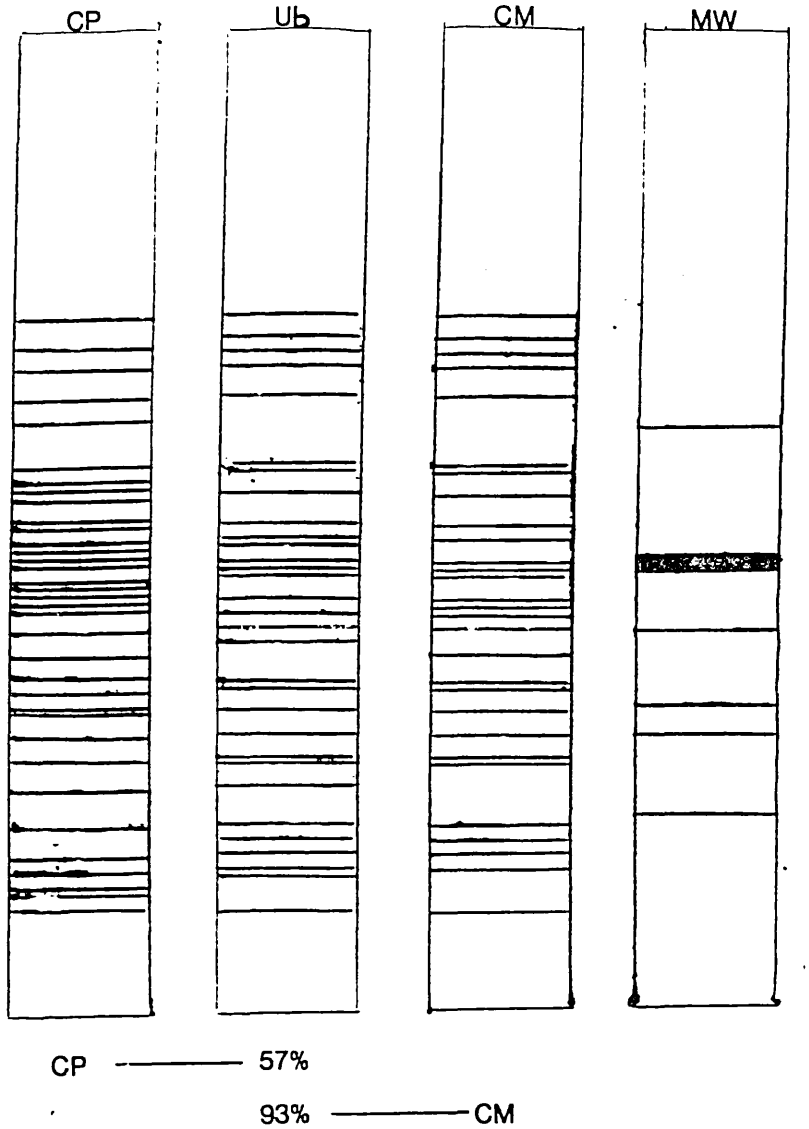


FIGURE 6.12. Diagrammatic map for the comparison of the protein profile of isolate Ub to the protein profiles of *C. puteana* and *C. marmorata*. The percentage similarities of isolate Ub to these standard organisms is indicated. CP - *C. puteana* FPRL 11E; Ub - isolate Ub; CM - *C. marmorata* FPRL 410; MW - Standard molecular weight markers (Figure 6.2.).

isolates were closely related to Ub, this result indicates that all isolates are *C. marmorata*.

6.2.2.2. DIRECT ANALYSIS OF SAMPLES WITHOUT MICROBIAL ISOLATION

Strand and mycelial samples removed from the *Unicorn* were analysed directly by SDS-PAGE. Samples Ui, Uk, Uj, Ul and Um were chosen for analysis due to the sizeable structures available and the profiles obtained (Figure 6.13.) are consistent with the samples being *C. marmorata*. The protein profile of sample Ui was also more similar to that of *C. marmorata* than to *C. puteana*. This is in contrast to the results shown in Figure 6.11., for the analysis of mycelia isolated from sample Ui. The percentage similarity index shown in Table 6.9., confirms the visual interpretation of results. The comparison of standard mycelial cultures of *C. puteana* and *C. marmorata* to the samples Ui-Um show a higher range of similarity values for *C. marmorata* than for *C. puteana* (42%-61% cf. 30%-55%). To further confirm the results samples of strand material (Ul) and field mycelial material (Um) share higher similarities with *C. marmorata* than with *C. puteana*. The index values obtained for this analysis are lower than values previously observed for the comparison of *C. puteana*/*C. marmorata* standards to mycelial isolates of these samples. Field to field comparisons however give higher values e.g. Ul and Um against the other isolates produce mean values of 72% and 62% respectively. Field material is therefore more appropriate to use as a reference than laboratory cultured material.

In this analysis Ui is again seen as somewhat ambiguous with evidence of lower index values for this sample in

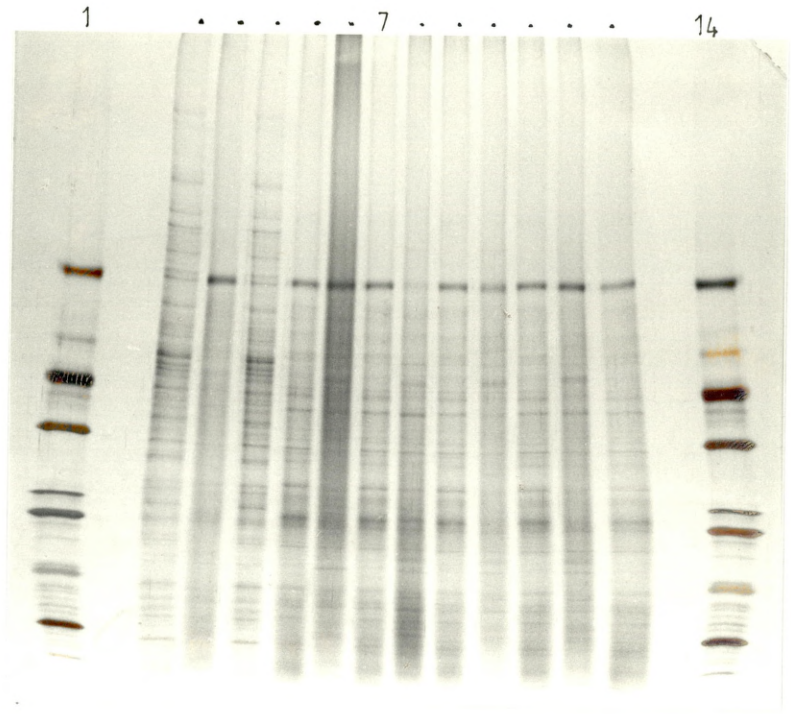


FIGURE 6.13. SDS-PAGE analysis of samples removed from the *Unicorn*. Tracks represent: 1, 14 - MWM (Figure 6.2.); 2, 4 - *C. puteana* FPRL 11E; 3 - isolate Ui; 5, 7, 9, 11, 13 - *C. marmorata* FPRL 410; 6, 8, 10, 12 - isolates Uk, Uj, Ul, Um.

TEST	CP	CM	Ui	Uj	Uk	Ul	Um
REF							
CP	100	55	30	53	48	43	48
CM	58	100	42	63	45	47	61
Ul	74	78	39	87	74	100	87
Um	68	82	43	71	61	71	100

KEY

CP, CM - Standard laboratory cultures of *C. puteana* and *C. marmorata*

Ul - Representative strand field sample

Um - Representative mycelial field sample

TABLE 6.9.

PERCENTAGE SIMILARITY INDEX FOR SAMPLES FROM THE *UNICORN* ANALYSED WITHOUT MICROBIAL ISOLATION

comparison to all the other samples (Table 6.9.). Since there is some confusion as to the identity of Ui, western blotting was carried out (Figure 6.14.). All samples including Ui share a similar antigen profile. However, although this profile is more similar to *C. marmorata* (tracks 2, 4, 6, 8, 10) than to *C. puteana* (tracks 11, 13) or to the antigen profiles of other basidiomycetes (chapter 3), it contains new antigenic species not present in the profile of laboratory cultured *C. marmorata*. The antigens indicated have a slightly higher molecular weight than comparable antigens present in the profiles of *C. marmorata*.

6.2.3. ANALYSIS OF CORE SAMPLES FROM THE *UNICORN*

Twenty-nine cores were removed from the forward hold of the *Unicorn* (Table 6.10.) in the locations subject to wet rot (detailed in Figures 2.5. and 2.6.) caused by the organism *C. marmorata* (as concluded in 6.2.2.1. and 6.2.2.2.). Each core was divided into 2 sections. One half was used for microbial isolation and subsequent SDS-PAGE, whilst the other was used as a source of antigens in dot immunoblotting and EIA studies. For microbial isolation, core sections were further divided in half depending on the length of the core (larger core lengths were available from Set A since sampling at this location was more accessible).

Table 6.10. indicates that from a total of 40 core sections, only 5 produced basidiomycete fungi in culture (12.5%). These isolated fungi were examined using SDS-PAGE in comparison to the standard reference organism *C. puteana* (Figure 6.15.). The protein profiles of the organisms share similarities with each other and with FPRL 11E (tracks 2,

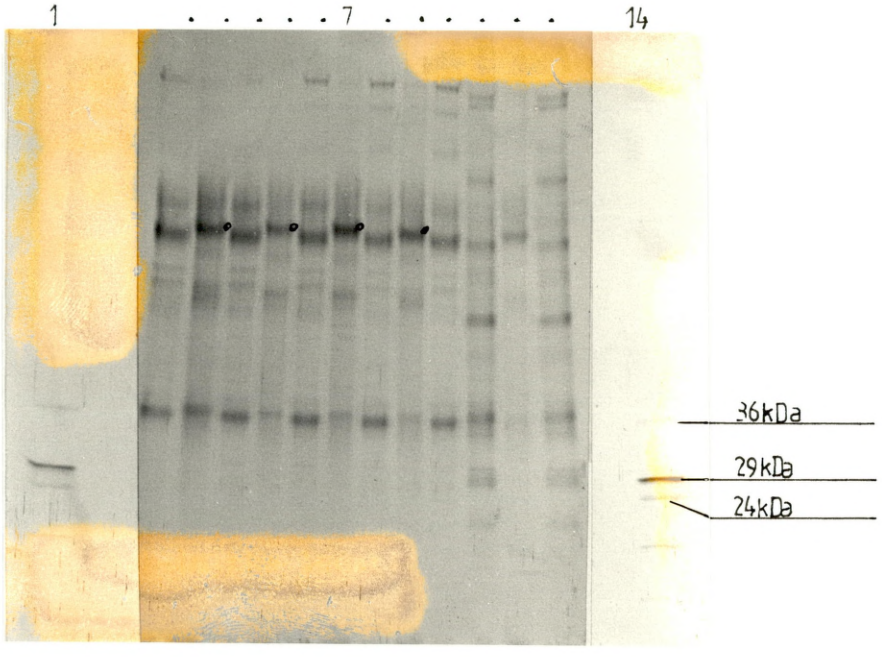


FIGURE 6.14. Western blotting analysis of samples removed from the *Unicorn*. Tracks represent: 1, 14 - MWM (Figure 6.2.); 2, 4, 6, 8, 10 - *C. marmorata* FPRL 410; 3, 5, 7, 9, 12 - isolates Um, Ul, Uk, Ui; 11, 13 - *C. puteana* FPRL 11E.

TABLE 6.10.

MICROBIAL ISOLATION OF ORGANISMS FROM CORES REMOVED FROM THE *UNICORN*

KEY Core sections 1-9 (Set A) were divided in two. a and b - Top and Bottom of core section. Core sections 10 and 16 (Set B) were also divided in two, whilst each of the remaining core sections of this set were analysed whole.

\+ growth; - no growth; ++ positive for clamp connections; -- negative for clamp connections; x not applicable.

SAMPLE	GROWTH OF FUNGI	BASIDIOMYCETE
SET A		
1a	+	++
1b	+	--
2a	-	x
2b	+	--
3a	-	x
3b	-	x
4a	-	x
4b	-	x
5a	+	--
5b	+	--
6a	-	x
6b	-	x
7a	+	--
7b	-	x
8a	-	x
8b	-	x
9a	-	x
9b	-	x

SAMPLE	GROWTH OF FUNGI	BASIDIOMYCETE
SET B		
10a	+	--
10b	+	++
11	-	x
12	+	--
13	+	++
14	+	--
15	+	--
16a	+	--
16b	+	--
17	-	x
18	-	x
19	-	x
20	-	x
21	-	x
22	-	x
23	-	x
24	+	++
25	+	--
26	+	--
27	+	++
28	-	x
29	+	--

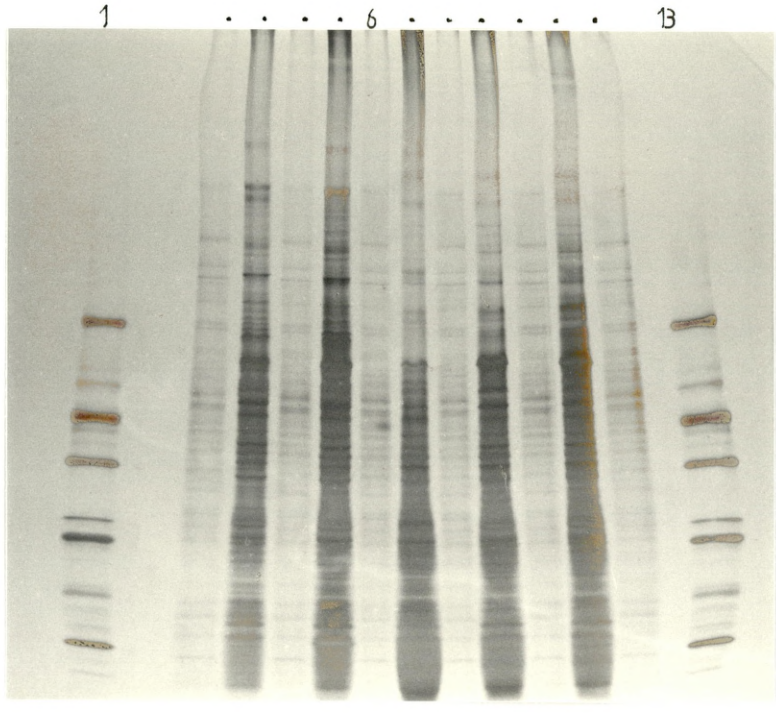


FIGURE 6.15. SDS-PAGE analysis of microbially isolated organisms from cores from the *Unicorn*. Tracks represent: 1, 13 - MWM (Figure 6.2.); 2, 4, 6, 8, 10, 12 - *C. puteana* FPRL 11E; 3, 5, 7, 9, 11 - Core isolates Set B, 27, 13, 24, 10b and 1a (SetA), respectively.

4, 6, 8, 10, 12; see also Table 6.11.). Using the core samples as references, the mean percentage range of similarity indices is 62%-72%, indicating that all the isolates are more similar to each other than to FPRL 11E (49%), but that they belong to the same genus, having similarities of >49%.

6.3. ANALYSIS OF FIELD SAMPLES USING A MONOCLONAL ANTIBODY

6.3.1. DOT IMMUNOBLOTTING

All field samples were analysed by dot immunoblotting using MAb 91/5B6 described in 5.5. EP extracts of *C. puteana* infected wood blocks were analysed. In addition, WM extracts of all mycelial isolates, of field strand and mycelial samples and of standard organisms, were prepared. Table 6.12., shows the plans of the immobilon (for dot immunoblotting) and microtitre plate (for EIA) used for the analysis.

Figure 6.16. exhibits the results of a dot immunoblotting analysis, with mean values for the intensity of the coloured spots, values of 2 or greater are considered positive). The MAb can detect *C. puteana* and *C. marmorata* at concentrations of at least 6.25µg per ml PBS, and can distinguish between different concentrations of *Coniophora* present. In addition, MAb 91/5B6 can detect *C. puteana* in extracts of infected wood blocks at weight losses at or greater than 0.94% (values of 3-4). In a limited cross-reactivity study (5.5.2.), the MAb was shown to be specific to the genus *Coniophora*, however, in the current analysis the MAb cross-reacted strongly with 6 samples from the *Unicorn* and *Discovery* and weakly with an additional 2

TEST	CP	27	13	24	10b	1a
REF						
CP	100	71	56	49	68	56
MEAN*	49	71	70	62	72	66

KEY

10b-27 - cores from Set B which produced basidiomycete mycelia during microbial analysis

1a - core from Set A which produced basidiomycete mycelia during microbial analysis

* - Mean percentage values for mycelia from cores 1a-27 when used as references

TABLE 6.11.

PERCENTAGE SIMILARITY INDEX FOR CORE ISOLATES

PLAN 1 - ANALYSIS OF *C. PUTEANA* INFECTED WOOD BLOCKS; WM EXTRACTS OF MYCELIAL ISOLATES, STANDARD ORGANISMS AND STRAND AND MYCELIAL FIELD SAMPLES

	CP		CPwb		MYCELIAL ISOLATES AND FIELD SAMPLES							
COL	1	2	3	4	5	6	7	8	9	10	11	12
ROW												
A	1:2		0%		U1		U20		Ug		Ul*	
B	1:4		0.94%		U2		DIc		Uh		Um*	
C	1:8		16.74%		U3		D3		Ui			
D	1:16		26.48%		U4		Ua		Uk			
E	1:32		34.12%		U51		Ub		Um			
F	1:64				U12b		Uc		Ui*			
G	CA				U17		Ud		Uj*			
H					U17b		Ue		Uk*			

PLAN 2 - ANALYSIS OF EP EXTRACTS OF CORE SECTIONS; WM EXTRACTS OF STANDARD ORGANISMS AND OTHER FUNGI KNOWN TO INHABIT THE *UNICORN*

	CORE SECTIONS								CM		FUNGI	
COL	1	2	3	4	5	6	7	8	9	10	11	12
ROW												
A	1		9		16a		23		1:2		SL	
B	2		10a		16b		24		1:4		AX	
C	3		10b		17		25		1:8		V	
D	4		11		18		26		1:16		PP	
E	5		12		19		27		1:32		LS	
F	6		13		20		28		1:64			
G	7		14		21		29					
H	8		15		22							

KEY

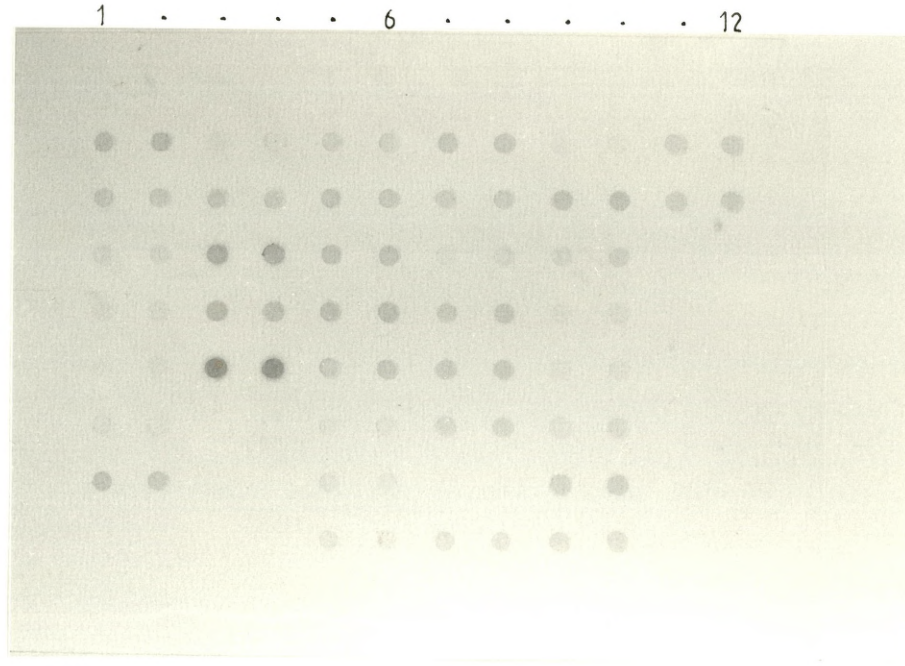
The initial concentration of CP, CA and CM is 50µg per ml PBS. 1:2-1:32 refer to doubling dilutions from the initial concentration. All samples were assayed in duplicate.

COL	-	Columns 1-12.
ROW	-	Rows A-H rows.
*	-	Strand and mycelial field samples.
wb	-	Infected wood blocks.
1-9	-	Set A. Whole core section analysed
10-29	-	Set B. Core sections 10 and 16 were divided into a further 2 sections prior to analysis.

TABLE 6.12.

SAMPLE PLAN FOR BOTH IMMOBILON AND MICROTITRE PLATE FOR ANALYSIS BY DOT IMMUNOBLOTTING AND EIA

A



B

PLAN 1 PLAN 2

	CP	CPwb	MYCELIAL ISOLATES/FIELD SAMPLES				CM
COL	1,2	3,4	5,6	7,8	9,10	11,12	11,12
ROW							
A	4	0	3	4	0	4	4
B	4	3	3	4	4	4	3
C	3	4	4	2	3		2
D	2	4	4	4	0		1
E	1	4	4	4	2		0
F	0		0	3	3		
G	4		0	0	4		
H			1	3	4		

KEY

PART A - Photographic observation
PART B - Mean values for coloured spots.
COL - Columns 1-12
ROW - Rows A-H
wb - Decayed wood blocks.

FIGURE 6.16. Dot immunoblotting analysis of field samples from the *Unicorn* and *Discovery* using MAb 91/5B6.

isolates. These organisms have been identified as *Verticillium* spp., *Poria* spp., *L. sulphureus* and *H. puberum*. However, the MAb did not cross-react with isolates Ud, Ug and Uk which were diagnosed as moulds (values of 0). Recognition of mycelial and strand samples of *C. marmorata* was possible producing values of 2-4. Dot immunoblotting analysis of the core samples from the *Unicorn* proved difficult due to the interference of coloured substances, probably phenolics, extracted from the decayed wood in the preparation of the antigen extracts. Consequently, EIA analysis was necessary.

6.3.2. EIA ANALYSIS

The plan detailed for the analysis of all samples (Table 6.12.) was utilised for the EIA examination using MAb 91/5B6 (Table 6.13.). Positive reactions with dilutions of *C. puteana* (PLAN 1) and *C. marmorata* (PLAN 2) at concentrations as low as 3.125µg per ml PBS were evident; a positive reaction was also found with *C. arida*. In addition, detection at all stages of decay tested, was confirmed. High reactivity with all *Coniophora* samples and isolates was evident. Although cross-reactivity of the MAb with some other basidiomycetes was apparent, the level of reactivity was much less than that observed for *Coniophora* samples. Further, only 4 isolates cross-reacted with the MAb in this system (identified as *Poria* spp. C5/D5, *H. puberum* B7 and *L. sulphureus* C7), compared to 8 organisms in the dot immunoassay. The cross-reactivity of the samples which was apparent in the EIA system was consistent with the cross-reactivity evident with laboratory cultured organisms (PLAN 2). Interestingly, the MAb did not cross-react with the moulds, Ud, Ug and Uk (ϛ7., A9 and D9:),

PLAN 1 - RESULTS FOR THE ANALYSIS OF *C. PUTEANA* INFECTED WOOD BLOCKS; WM EXTRACTS OF STANDARD ORGANISM; WM EXTRACTS OF FIELD STRAND AND MYCELIAL SAMPLES

	CP		CPwb		MYCELIAL ISOLATES AND FIELD SAMPLES							
COL	1	2	3	4	5	6	7	8	9	10	11	12
ROW												
A	27.5		1.0		-		**28.9**		-		**13.9**	
B	23.2		4.0		-		2.3		**18.9**		**17.4**	
C	20.9		10.8		3.9		2.8		**15.2**		-	
D	14.8		18.5		5.9		**22.6**		-		-	
E	10.9		25.7		-		**24.0**		**19.7**		-	
F	2.5		-		-		**24.0**		**14.5**		-	
G	25.0		-		-		-		**27.7**		-	
H	-		-		-		**25.4**		**19.6**		-	

PLAN 2 - RESULTS FOR THE ANALYSIS OF EP EXTRACTS OF CORE SECTIONS; WM EXTRACTS OF STANDARD ORGANISMS AND OTHER FUNGI KNOWN TO INHABIT THE *UNICORN*

	CORE SECTIONS								CM		FUNGI	
COL	1	2	3	4	5	6	7	8	9	10	11	12
ROW												
A	2.7		-		-		-		16.8		-	
B	-		-		2.3		-		13.8		4.2	
C	-		3.3		-		-		11.9		-	
D	-		-		2.3		-		8.7		3.8	
E	-		-		-		2.4		5.3		2.0	
F	-		-		3.3		-		4.2		-	
G	-		-		-		-		-		-	
H	-		2.9		-		-		-		-	

KEY

Each value represents the average OD value divided by the OD value of uninfected wood blocks (0.054). The control assay without MAb produced values less than 0.054 and is therefore not included.

\- - Values less than or equal to 0.054.
COL - Columns 1-12
ROW - Rows A-H

N.B. **BOLD** values in PLAN 1 represent *Coniophora* samples

TABLE 6.13.

EIA ANALYSIS OF ALL SAMPLES

suggesting that although the MAb has some cross-reactivity it may be confined to the members of the basidiomycotina.

Unlike the dot blot immunoassay, no problems were encountered with the analysis of decayed core samples using the EIA system. Positive reactions, although weak, were observed for the following core sections, 1, 10b, 15, 16b, 18, 20 and 27. Of these samples, basidiomycete mycelia was grown from section 1a of core 1, section 10b of core 10 and core 27. Cores 13 and 24 which also produced basidiomycete mycelia were positive in the assay and the identity of the organisms within cores 15, 16, 18 and 20 have not yet been established. Further research however to reduce the cross-reactivity of the MAb and to increase the signal for positive reactions in field samples is necessary. The remaining samples which exhibited positive reactions, produced no viable basidiomycete mycelia during microbial analysis. This suggests that it may be possible to detect members of the *Coniophora* genus in wood, regardless of the viability of the organisms.

6.5. DISCUSSION

6.5.1. IDENTIFICATION

The research discussed in chapter 3, dealt with the identification of laboratory cultured *C. puteana* and related organisms using the techniques of SDS-PAGE and western blotting. The recognition of unique protein and antigen profiles for individual organisms allows identification. The research detailed in this section describes the ability to identify fungal samples from the frigate *Unicorn* and RRS *Discovery*, using SDS-PAGE and western blotting.

Methods for the identification of wood decay fungi can be divided into primary and secondary systems. For all identification systems the primary method involves the visual examination of several features associated with decay. The first is the presence of fruiting body structures which in most situations allow identification, as was the case in this current study with the fruiting body of *L. sulphureus*. The second is the nature of any mycelial or strand material present which may assist in determining the identity of an organism. Finally, the third feature is the appearance of the timber, which can provide information on whether the decay is caused by brown, white or soft rot fungi. Primary methods however do not provide definitive identification in all cases and therefore secondary methods are necessary.

The secondary methods involve the study of mycelia isolated from field samples by a variety of techniques. The conventional method of identification involves the analysis

of mycelia with reference to various keys which together provide information on such features as hyphal width, size of hyphal spores, production of acids and release of enzymes from mycelia (Nobles, 1965; Stalpers, 1978). Although an extensive range of secondary tests are available which aid in the determination of the identity of decay fungi, the results obtained may turn out, in due course, to be incorrect. Additional secondary identification methods are therefore required. Recently the molecular techniques of SDS-PAGE and western blotting have been used by a number of researchers studying wood decay fungi (Schmidt and Kebernik, 1989; Palfreyman *et al.*,1991a, 1991b; and M^{c}Dowell *et al.*, 1992a and 1992b). These techniques allow a novel method of secondary identification, using macro-molecules as opposed to whole organisms. In addition, the molecular methods may lead to simpler methods, eg. identification using an organism specific monoclonal antibody.

The molecular methods of SDS-PAGE and western blotting however, can only allow the recognition of profiles for identification if the appropriate reference organisms are available. For example, in this study, no indication of the identity of isolates U12b, U17, U17b, U1, U2, U13, D3f and DIc was found using electrophoretic techniques (Table 6.3.a.), since none of the protein profiles of the reference organisms in the culture collection at Dundee Institute of Technology matched the protein profiles of these isolates. If reference organisms are available, SDS-PAGE can be used to positively identify individual wood decay fungi; confirmation of identity can be made by high percentage similarity index values, as was possible for isolates U4, U52, U61, U20 and D3 (Table 6.6.).

Several unidentified isolates were analysed conventionally at CBS Baarn. These isolates were subsequently examined using SDS-PAGE in comparison to reference fungi indicated by and obtained from CBS Baarn. The identity of isolates D3f, DIc, U13 and U2 was confirmed, highlighting the use of SDS-PAGE as an identification system. The conventional analysis of isolate U1 at CBS Baarn indicated that this isolate was *Verticillium* cf. *lamellicola* (which implies that classification is not certain; Watling, personal communication). A percentage similarity index value of 50% was obtained by an SDS-PAGE comparison to the reference organism *V. lamellicola*, also suggesting that the identification of isolate U1 is uncertain.

Definitive identification using both SDS-PAGE and conventional methods may not always be possible. For example, isolate U17b was identified as *P. igniarius* at CBS Baarn, but its suggested identity by SDS-PAGE was *V. lecanii*. The SDS-PAGE identity was supported by a higher percentage similarity index value of 67% for *V. lecanii* cf. 50% for *P. igniarius*. These results indicate that either the conventional identity of this isolate was inaccurate or that SDS-PAGE alone cannot discriminate between certain reference fungi. Further analysis of SDS-PAGE supplemented with western blotting may aid identification.

Whilst it seems likely that SDS-PAGE identification is limited by the availability of appropriate reference organisms, expansion of cultures collections for molecular studies is essential in order to demonstrate this conclusively. The identity of isolate U9 was not certain because four reference organisms exhibited relatively high

index values in comparison to this isolate (ie. FV -50%, PP - 50%, AX - 50%, PI - 68%; Table 6.6.). These results may suggest the lack of appropriate reference organsisms but the variability may also be due to the discovery of a new organism or at least a new strain of an organism.

A further limitation of SDS-PAGE is that low similarity index values may not be related to lack of reference organisms, but to the molecular variability of particular isolates. For example the strains of *C. puteana* examined in chapter 3, were more variable than those of *S. lacrymans* (Palfreyman *et al.*, 1991). Other decay fungi may exhibit even greater variation than *C. puteana*, eg. isolate U9, thus rendering identification by SDS-PAGE more difficult and in some cases identification may be impossible.

The secondary identification methods involved the examination of isolated mycelia. Such microbial isolation has associated problems. For example, in the course of isolation it is not always possible to obtain a pure culture of a basidiomycete organism due to growth inhibition by moulds such as *Penicillium* and *Trichoderma*. In addition, isolation depends on the viability of the organism, thus mycelia cannot always be cultured from decayed wood, fruiting bodies or strand material. Further it may happen that more than one basidiomycete is isolated from the same fruiting body sample, making it impossible to determine the identity of the organism which produced the fruiting structure. For example, the fruiting body sample U5 produced two types of basidiomycete organism, one of which was positively identified as *P. placenta*, whilst the identity of the other remains uncertain (Table 6.3.a).

Analysis of field samples without the need for mycelial isolation would overcome these problems and is therefore more attractive for identification purposes. Evidence that such identification is possible using SDS-PAGE is provided in this chapter by the analysis of strand and mycelial samples of *C. marmorata*. Similarly, limited numbers of mycelial and basidiocarp field samples of *S. lacrymans* have successfully been identified by direct examination using SDS-PAGE (Vigrow, 1992).

However for both *C. puteana* and *S. lacrymans*, the profiles of field specimens such as strand samples are less similar to laboratory standards, than are isolated mycelial samples. Changes in profiles may be due to a lower protein content, indicative of the collapse, lysis and reprocessing of constituents of hyphae (Jennings and Watkinson, 1984). Evidence for this reprocessing may be exhibited by the antigen profile of the strand material of *C. marmorata*. Although similar to the profile of laboratory cultured mycelial material of the same organism, the profile of the strand material exhibited new antigenic species and molecules which shared similarities to the mycelial profile, but which may represent reprocessed antigens.

For identification of field samples by SDS-PAGE the most appropriate standard would be field material and thus a database of such samples is required. The results in this chapter for *C. marmorata* and those described for *S. lacrymans* (Palfreyman *et al.*, 1991), provide some evidence that field-field comparisons are more reliable, showing a greater resemblance in profiles and thus higher similarity indices. Specific comparisons of strand-strand and mycelial-mycelial samples may be necessary for increased

accuracy. Despite this however identification may not always be possible. For example, the identity of field sample Ui was not confirmed in this study, since the profile of an extract of the field sample was similar to *C. marmorata*, whilst the profile of an extract of the isolated mycelia was similar to *C. puteana*.

6.5.2. DETECTION

It has been demonstrated that molecular methods can be used to identify organisms colonising wood in the field. Since these methods have indicated that individual organisms have unique molecular profiles the development of simple antibody-based systems for the detection of organisms in wood is feasible. For a simple detection system, a simple assay is essential, eg. dot immunoblotting and/or EIA. In addition specific probes viz., monoclonal antibodies (MAbs), may be required for the detection of particular organisms.

The ultimate requirement of an antibody-based detection system for wood decay fungi is to detect the organisms prior to structural damage of wood, in order to establish the early presence of organisms for subsequent preservative treatment. In addition some immunological systems have the potential for the development of on-site techniques which would provide monitoring systems to determine the presence of fungi in timbers at all stages of decay.

In this study, dot immunoblotting and EIA using a MAb were analysed for the detection of *Coniophora* in the field. A comparison of the two systems will now be discussed with

reference to Figure 6.17. In addition, the use of a MAb in these systems will be discussed.

The dot immunoblotting technique was applied for the detection of members of the *Coniophora* genus, since this technique offers the possibility of future development to an on-site DIP-STICK immunoassay for detection, e.g. for the detection of *Humicola lanuginosa* in rice grains (Dewey *et al.*, 1989). Although it was possible using dot immunoblotting with a MAb to detect *C. puteana* in laboratory decayed wood samples, and strand and mycelial samples of *C. marmorata* in the field, the application of this system to field wood samples was not possible since the coloured wood extract produced bound to the immobilon membrane, preventing interpretation of the results.

Since dot immunoblotting cannot be used for the analysis of field wood samples, and EIA was shown to be effective in allowing the detection of varying levels of antigens in *C. puteana* decayed wood blocks (chapter 5), EIA was tested using field samples. The highly coloured extracts of the field samples did not interfere with the EIA. This is most likely due to the lower efficiency of the plastic support of the EIA in retaining bound components during washing procedures, than the dot immunoblotting immobilon membrane, thus reducing binding of the extract components. The lower binding capacity of EIA is demonstrated by the following example using IgG; 400ng/cm^2 IgG can be bound in EIA; Gibco Ltd., whilst 172mg/cm^2 IgG can be bound in dot immunoblotting; Millipore Ltd.).

The limited field study carried out using EIA, indicated that most core samples which produced positive reactions in

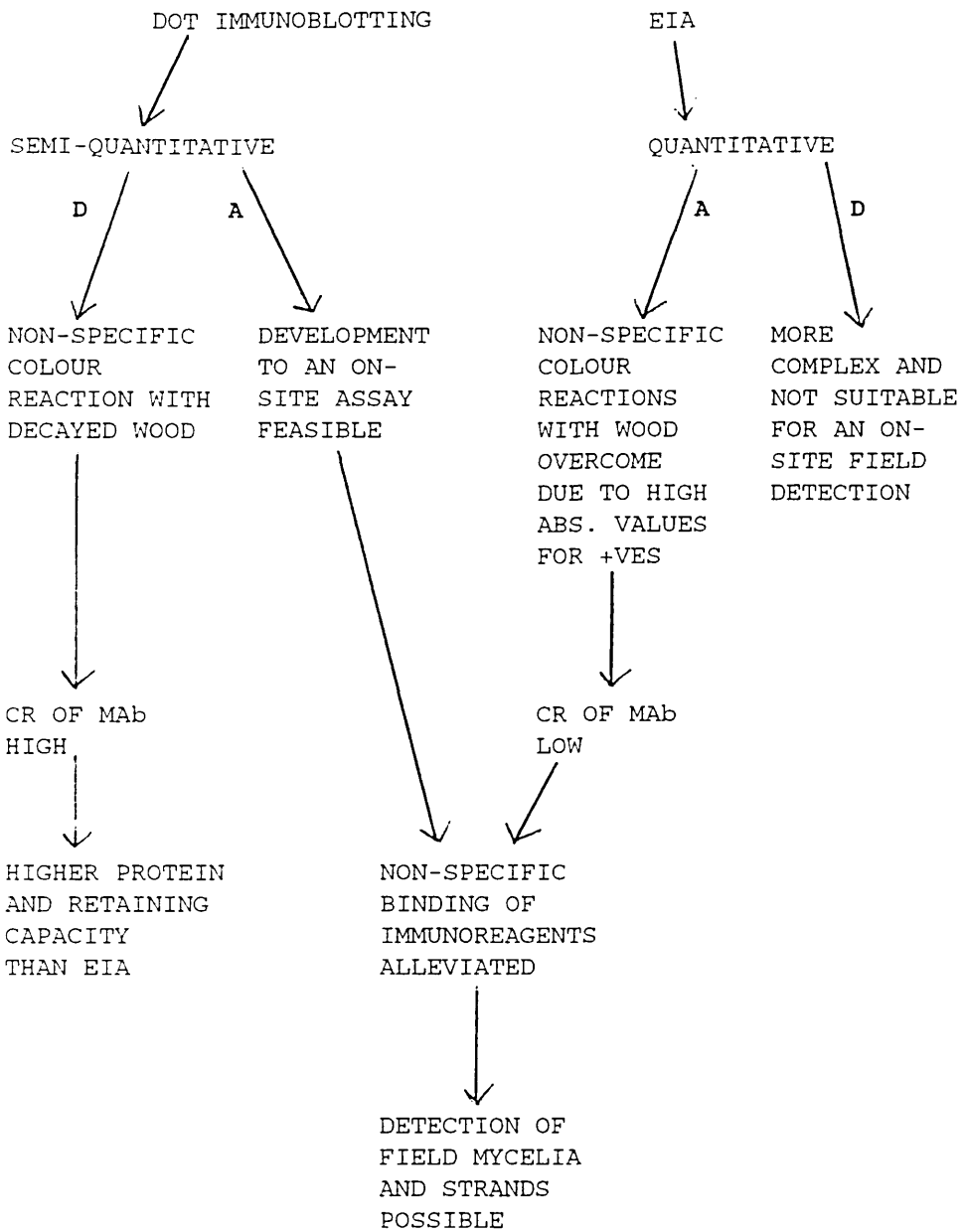


FIGURE 6.17. Comparison of the Dot immunoblotting and EIA systems, and their application to field samples using MAb 91/5B6. (**D** - Disadvantages; **A** - Advantages).

EIA were also positive by microbial isolation, the organism isolated being identified as *C. marmorata*. It was also possible to detect, by EIA, *Coniophora* in core samples which produced no viable mycelia. Since the MAb exhibited a degree of cross-reactivity (see later), it can be argued that these positives could relate to any organism, but given the abundance of *C. marmorata* in the sampling areas of the ship, this seems unlikely. Although detection of *C. marmorata* in these samples is apparent, a feasibility study of a more extensive range of field samples of *Coniophora* is required, before a conclusive statement as to the efficiency of EIA as a method of detection of *Coniophora* in the field can be made.

The production of MAbs to wood decay fungi has only been reported in recent years and their application is currently limited to only a few additional basidiomycete organisms viz., *P. placenta*, *P. chrysosporium* (Jellison and Goodell, 1986; Clausen *et al.*, 1989; Clausen, 1991; Daniel *et al.*, 1991), *S. lacrymans* and *H. annosum* (Glancy personal communication; Galbraith, PhD. thesis in preparation).

MAb 91/5B6 described in detail in chapter 5, was found to be specific to the genus *Coniophora* in limited cross-reactivity studies. However the application of this MAb to field samples indicated some cross-reactivity of the MAb to WM extracts of mycelial isolates and EP extracts of reference organisms (dot immunoblot data, Plan 2, Figure 6.16., not shown). The cross-reactivity of the MAb was lower using EIA in comparison to the dot immunoblotting system. This implies that the level of cross-reactivity of the MAb is a feature of the immunoassays themselves, due to the higher binding capacity of the dot immunoblotting

system. Despite the cross-reactivity, the MAb alleviated problems of non-specific binding of immunoreagents apparent with polyclonal antisera and reacted strongly with strand and mycelial samples of *C. marmorata*. These results are summarised in Figure 6.17.

It should be possible however to produce a MAb which is more specific to *Coniophora* organisms by the use of the unique EAs revealed in this study, as immunogens. The specificity of polyclonal antisera has been improved by the use of specific proteins isolated from SDS-PAGE gels, as immunogens (Kroll, 1981). Such a method is feasible for *Coniophora* using particular EAs. In addition, the use of immunomodulators such as cyclophosphamide is possible for *Coniophora* organisms, since their use has been described for the production of more specific MAbs to *H. capsulatum* (Hamilton *et al.*, 1990) and to the forest pathogen *H. annosum* (Galbraith, PhD. thesis in preparation) by the suppression of the immune response to cross-reacting organisms.

The reactivity of the MAb developed in this project indicated a stronger reaction with WM antigens than with EAs. The increased reaction suggests that epitopes present on WM antigens recognised by the MAb are in greater quantities than those on EP antigens, and this may be confirmed by the greater reaction of the MAb with the WM extracts of decayed wood samples, compared to EP extracts of the same samples (5.5.3.). The fact that the MAb (produced from EAs) has reacted with WM antigens at all, suggests that it is recognising epitopes on the mycelial wall of these organisms which are perhaps precursors to exoantigens. This suggests therefore that the WM antigens

may be more useful in detection systems for *Coniophora* than EAs due to the greater signal produced, though the preparation of EAs is simpler.

The cross-reactivity of the MAb to EA preparations of non-*Coniophora* organisms was lower than that for WM preparations of isolates and may simply be due to the epitope concentration effect previously described. However, since there are few EAs which are common to decay fungi in comparison to WM proteins (chapter 3) it is possible that the cross-reactivity of the MAb with EP extracts is less than that with WM extracts, due to the specificity of the EA/EPs. If this is the case, then EAs would be more useful than WM antigens for a detection system. Further analysis is therefore required to determine whether the cross-reactivity is actually lower for EAs or if the different levels of detection are simply due to concentration effects.

If a low level of cross-reactivity to EP extracts is genuine for both dot immunoblotting and EIA, then this result is not unusual. For example, Dewey *et al.*, (1989) described a relatively specific MAb used to detect the fungal organism *H. lanuginosa* in rice grains. This MAb cross-reacted strongly with only one test fungus and partially with two others. Further research is therefore now required to substantiate the results for *Coniophora*.

CHAPTER 7

THE POTENTIAL IMPORTANCE OF MOLECULAR STUDIES ON FUNGI, TO MARITIME ARTIFACTS

In recent years there has been a world-wide re-awakening of interest in the location, recovery and conservation of wooden ships. Such artifacts are of substantial significance to historians, but most importantly they allow the history of their era to become known. Furthermore access to certain historic ships is allowed for the pleasure of the general public. Details of maritime artifacts which are currently open to the public are provided in chapter 1, however, two artifacts, the frigate *Unicorn* and RRS *Discovery*, highly relevant to this present study, require further discussion.

The frigate *Unicorn* and RRS *Discovery* are currently under the protection of the *Unicorn* Preservation Society and Dundee Industrial Heritage Ltd., respectively, and are both subject to wet rot infections. Since both ships are major tourist attractions for the City of Dundee, and the *Discovery* is the flagship of Dundee's economic recovery, the research program described in this thesis was designed as an adjunct to the long term conservation of these artifacts.

The frigate *Unicorn* was 168 years old in 1992. She was constructed in the early nineteenth century at the transition between the design of wooden clad and iron clad warships (Goodwin, 1987). She was launched in 1824 for the Royal Navy, and since this period was of settled peace, she was immediately roofed and laid up in reserve and so she remained. As a result of her sheltered existence she is in excellent condition for her age and is rated as one of the top ten warships worldwide which are still afloat (*Unicorn* Preservation Society, 1985). There is however an inherent problem of decay in this historic ship which requires to be

treated if she is to remain in her present "original form", and provide such exciting history of the period in which she was built.

The Antarctic Exploration ship, *Discovery* (re-named RRS Discovery in 1925), is a prime exhibit of the type of ships which were used for the discovery of unknown lands. The skilled craftsmen and women of Dundee won the tender for the design and assembly of the *Discovery* at the beginning of the twentieth century as a result of their successful whaling vessels. The *Discovery*, launched in 1901, was a successor to Captain Cook's ships, the *Resolution* and the *Adventure* and is a survivor from the "heroic age" of Antarctic exploration in the early years of the twentieth century. She has a unique basket-like structure and three-skinned timber composition which was designed to survive pressure from the ice in Antarctica. This design was so successful, that she was able to withstand being trapped in the ice for two whole years, before being released in the Antarctic summer of 1904. Her unique structure is however conducive to decay, but *Discovery* has survived, in spite of her design, due to her historical connections.

In practice, unsophisticated, but effective methods for the detection of fungal decay which are used for building timbers are also those used for detecting fungal infections on historic artifacts such as the *Unicorn* and *Discovery* (chapter 1). These techniques however provide evidence of fungal decay when the only solution to contain the infection, is the removal and replacement of suspect timbers. As detailed in chapter 1, additional methods for the detection of fungal decay exist (Friss-Hansen, 1980; Line, 1981, 1982; Baldwin and Streisel, 1985; Gibson *et*

al., 1985; Morrell *et al.*, 1986; Niku-Paavola *et al.*, 1990; Lawrence and Nilsson, 1991). However these methods rely on the detection of either physical or chemical changes of the wood which are associated with decay. These techniques cannot detect decay at an early stage to then allow sufficient preservative treatment and the methods only provide data on whether the timber needs replacing.

With regard to the timbers found on the *Unicorn* and *Discovery*, replacement is not always feasible. The unique structure of the *Discovery* renders particular timbers within that construction impossible to replace without the need for massive rebuilding, which in economic terms is unrealistic. Much of the accessible timbers of the *Discovery* which exhibited wet rot infections have already been replaced, whilst such timbers on the *Unicorn* have important historical value and replacement would result in the loss of precious historical detail. For example, many of the timbers of the *Unicorn* have engraved markings which represent a builders' map of where and when particular timbers were fitted during the construction of the ship. Such information provides details of the way in which construction of such ships were actually carried out. In addition, the Royal Architects, although setting down relatively strict guidelines of how a ship should be built, allowed some initiative from the builders; consequently, much of the construction of the *Unicorn* was at the builders' discretion (Goodwin, 1987).

A variety of methods have been proposed to identify and detect fungal organisms causing decay in any wooden construction. Such methods include the detection of organisms using isolation techniques followed by

identification based upon morphological characteristics (Nobles, 1965). These methods however are time consuming and require expert analysis. In recent years, the potential of molecular and immunological analysis of wood decay fungi has been realised, as a result of which, identification and detection problems are certainly simplified and can be overcome. The number of research reports describing such techniques reflect their increasing use in the mycological analysis of wood decay (Goodell and Jellison, 1986; Breuil *et al.*, 1988; Glancy, 1990; Schmidt and Kebernick, 1989; Palfreyman *et al.*, 1987, 1988, 1991; Vigrow *et al.*, 1991; M^{c}Dowell *et al.*, 1992a, b). The application of these techniques to the timbers of the frigate *Unicorn* and the RRS *Discovery* is described.

The assessment of the timbers on the historic ships, in particular the *Unicorn*, using the SDS-PAGE and western blotting techniques for identification purposes, indicated that there was a more substantial range of wet rot basidiomycete fungi colonising the timbers, than might have been predicted. Whilst it is appreciated that some of the basidiomycetes might be of marine origin since the timber may have become moist due to ingress of sea water, particularly in the hold of the ship, this seems unlikely, due to the isolation procedures used (Hunt and Cobb, 1971). In addition, the isolation procedures revealed only wood decay basidiomycetes, the existence of marine organisms in the timbers of the hold being highly unlikely due to the variation in salinity levels of the bilge water, as a consequence of the external environmental conditions (M^{c}Cutcheon, personal communication). Since a large number of basidiomycetes were isolated from the hold and that very few basidiomycetes have been isolated from marine

environments (Kohlmeyer and Kohlmeyer, 1979), this further suggests that the ecological niche within the timbers of the *Unicorn* does not support the growth of marine fungi.

Since *C. puteana* is the major wet rot organism which causes decay of wood in buildings in the United Kingdom (Coggins, 1980), it was predicted that this organism would be the primary cause of decay on the *Unicorn*. However, the predominant wet rot organism was *C. marmorata* and not *C. puteana*. Consequently the occurrence of *C. marmorata* was unexpected but Ginns speculated in 1982 that since *C. marmorata* occurs in domestic habitats, many outbreaks of fungal decay in buildings in the United Kingdom may have been mistakenly identified as *C. puteana*, when they may have been due to *C. marmorata*. Further, Bech-Anderson (1992) has reported that *C. marmorata* is found in connection with stone and cement foundations and that there is some evidence (not provided by Bech-Anderson) which suggests that *C. marmorata* is dependant on a calcium source for the neutralisation of oxalic acid, as is *S. lacrymans* (Jennings, 1991).

C. marmorata has been extensively located attacking timbers only in the hold of the *Unicorn*. Since the environment in which this organism is growing contains only wood and no stone or cement which would provide a calcium source, the results described strongly suggest that *C. marmorata* is not dependant on a calcium source for growth.

The evidence indicated by Bech-Anderson (1992) on the utilisation of calcium by *C. marmorata* may therefore be inaccurate. This organism may utilise calcium for neutralisation of oxalic acid. It is likely that a calcium

source is useful but not essential for growth, the organism having the ability to buffer its substrate by the production of different organic acids in a similar manner to *C. puteana* (chapter 1). The growth of *C. puteana* is not inhibited in the absence of a calcium source (chapter 1; Jennings, 1991). Experimentation to investigate the calcium requirements of *C. marmorata* need to be addressed. Simple culture tests varying the calcium content of the medium would be the first step to determine the effect of varying calcium levels. This would be particularly important to extend to tests using infected wood blocks and a natural calcium source such as stone or cement.

In addition to *C. marmorata*, other decay fungi have been identified from the timbers of the *Unicorn*. The variety of organisms may be a consequence of the unique environment of the *Unicorn* which is constructed primarily of wood. In addition, some decay may be due to use of construction timber from infected trees. As long as the timber remains moist the organisms can continue to proliferate, a problem evident in many wooden ships due to inadequate design and maintenance (chapter 1). For example, *L. sulphureus* is reported as being the most serious cause of decay of parkland oaks and large structural timbers such as those on wooden ships (Cartright and Findlay, 1958). This organism was discovered attacking oak timbers in a cabin on the *Discovery* and thus there is the possibility that this organism had already colonised the timber used in initial construction.

The distribution of wood decay fungi within the timbers of the *Unicorn* may be related to moisture levels. For example, *C. marmorata* can survive in timber with a high moisture

content, and has not been found outwith the hold area of the *Unicorn*. Specifically, *C. marmorata* has been identified from the keelson and surrounding timbers. These timbers all show excessive moisture levels (M^{c}Cutcheon, personal communication). Other organisms, for example, *Poria* spp., and *Verticillium* spp., which require lower moisture levels than *Coniophora* organisms (Cartwright and Findlay, 1958), have been found in timbers at the periphery of the hold, which have elevated moisture levels, but not as high as the keelson and surrounding timbers (M^{c}Cutcheon, personal communication). Further analysis to determine whether there is a definite relationship between organism location and moisture levels is required. This information may enable predictions of areas most at risk from decay by particular organisms and thus appropriate preservation regimes may be applied.

In addition to moisture levels, the occurrence of particular organisms may be related to timber type. For example, *L. sulphureus* found on oak timbers in the *Discovery* was also responsible for extensive decay of the oak timbers of the HMS *Victory* (Desch and Dinwoodie, 1981; Cartwright and Findlay, 1958). Species of the white rot organism *Phellinus*, also known to attack oak were isolated from oak timbers of the *Unicorn* and were recently found in the oak fore-mast of the *Vasa* ship that sank off Stockholm in 1628 (Nilsson and Daniel, 1992) and organisms such as *Poria*, *Coniophora* and *Verticillium* are also reported to grow on oak, the most abundant wood type on the *Unicorn*.

An aim of this current study was to develop immunological detection systems, specifically for *C. puteana* in wood which would allow detection prior to stuctural damage. The

application of these systems to the *Unicorn* and *Discovery* should also be possible. A summary of the successes and failures of the detection systems developed will now be discussed with reference to Table 7.1. Three polyclonal antisera and a monoclonal antibody were assessed for their ability to meet the requirements of Table 7.1.

Three systems were studied, western blotting, dot immunoblotting and EIA. Western blotting allowed the detection of *C. puteana* in wood blocks at most stages of decay using 3 polyclonal reagents. Only one of these reagents, antiserum 88/1 (raised against WM extracts of *C. puteana*) allowed the detection of antigens at very low weight losses, and thus prior to structural damage of the wood. Detection of *C. puteana* using this system was possible since the antigens detected are unique to the organism. Although meeting most of the requirements detailed in Table 7.1., western blotting is a complex technique to be used as a detection system, therefore alternative methods were examined.

Simpler immunological methods include dot immunoblotting and EIA. The former technique was rendered useless as an assay for detection using polyclonal antisera due to the ability of the antisera to bind non-specifically with other reagents. The latter technique, EIA, however, did allow the detection of *C. puteana* in wood blocks at all stages of decay and prior to structural damage, although specific detection of *C. puteana* was not possible.

Since the polyclonal antisera were not specific to *C. puteana*, the only technique which could detect specific decay fungi isolated from wood from the field, was western

REQUIREMENTS OF ASSAY

1. To detect CP/CM in wood.
2. To detect CP/CM in wood prior to structural damage.
3. To specifically detect CP/CM in wood.
4. To detect CP/CM in wood at all stages of decay.

	LAB TEST			FIELD TEST		
	WB	EIA	DB	WB	EIA	DB
1	//	//	x	/	/	x
2	/	/	x	xp	xp	x
3	//**	x	x	/	x	x
4	//	/	x	xp	xp	x

KEY

CP - *C. puteana* in lab tests
CM - *C. marmorata* in field tests
WB - Western blotting
EIA - enzyme immunosorbent assay
DB - Dot immunoblotting
// - tested positive with all reagents
/ - tested positive with some reagents
x - tested negative
** - linked to identification
p - possible

TABLE 7.1.

ASSESSMENT OF ASSAY REQUIREMENTS

blotting. The application of western blotting to cores removed from the *Unicorn* therefore allowed the detection of *C. marmorata* within certain timbers of the *Unicorn* by the recognition of a specific antigen profile of *C. marmorata* mycelia. The timber examined provided visual evidence of decay by the presence of crumbling wood and in some cases fungal growth on the surface of the timber, although cores removed were relatively sound. It seems plausible to suggest that due to the success of the western blotting in detecting *C. marmorata* grown from wood cores that this system could be used in the future to detect *C. marmorata* or indeed other decay fungi in wood cores without the need for microbial isolation, by the recognition of specific antigens.

More specific reagents were therefore investigated and monoclonal antibodies to *C. puteana* were produced. This reagent did exhibit some cross-reactivity, reacting with members of the genus *Coniophora* and some other decay fungi, but remained more specific than the reagents previously developed for *C. puteana*. The dot immunoblotting and the EIA systems were subsequently applied to decayed wood blocks using a monoclonal antibody probe. Both systems were capable of detecting *C. puteana* in wood, although the EIA also showed the ability to detect *C. puteana* at low weight losses, prior to structural damage of the wood. Consequently, EIA appears to be the most favourable technique to detect *C. puteana* in wood at an early stage in the decay process. The application of the dot immunoblotting system to the *Unicorn* was not possible due to the highly coloured antigen extracts which interfered in the assay. The EIA system however was applicable to the *Unicorn* detecting *C. marmorata* in wood cores, but detection

was not possible at all stages of decay, or prior to structural damage.

The requirements detailed in Table 7.1. were not completely fullfilled by any of the systems developed. However using the available reagents in an EIA it should be possible with a more extensive study to detect *C. marmorata* at all stages of decay in the timber of the *Unicorn*. It is possible that false positives may be apparent with this assay system and if the requirements are to detect a particular organism, such as *C. marmorata*, all positives should be checked by identification and detection of the organisms within wood cores by direct examination using SDS-PAGE and western blotting.

For the purpose of conservation of the *Unicorn* and *Discovery* it may not be necessary to develop a system specifically for one fungal species, but to have a system available which can detect general basidiomycete colonisation. From this project such a system is available using monoclonal antibodies or polyclonal antisera in conjunction with the EIA technique. The efficiency of the EIA system to detect fungal colonisation on the *Unicorn* is now considered in comparison to detection methods detailed in chapter 1. From the methods described, the following, have the same ability as EIA to provide quick sample analysis and to detect incipient decay, Computed Axialtomography, Infrared spectrophotemetry, Differential scanning calorimetry, Ion Mobility Spectrometry and Plant lectins. With the exception of detection by plant lectins all the techniques require expensive equipment such as spectrophotometers. Only 2 techniques, EIA and the CAT scan have been tested using field samples and of these, EIA is

the one with the capability of distinguishing between different fungi associated with decay. If the detection of specific organism is required, eg. to distinguish between early infections of wood by *C. puteana* and *S. lacrymans* then EIA is the most favourable since the equipment required for EIA is less expensive than that required for the CAT scan. However, as previously indicated the detection of a colonising basidiomycete may be all that is required, in which case the remaining techniques, infrared spectrophotometry, differential scanning calorimetry, ion mobility spectrometry and the use of plant lectins, in addition to EIA with a non-specific antiserum, can be employed. Owing to the cost of the equipment required however, EIA and plant lectins are more favourable in economic terms.

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APPENDIX 1

SOLUTIONS

APPENDIX 1 - SOLUTIONS

All chemicals used were from Sigma Chemical Company Ltd., unless otherwise stated. All solutions were stored long term at -20°C unless otherwise indicated and those solutions denoted by # were prepared only when required.

PART A - SOLUTIONS FOR EIA

1. PBS; 10mM, pH 7.4

SOLUTION A

Sodium dihydrogen phosphate	2.964g
Distilled water	950ml

SOLUTION B

Disodium hydrogen phosphate	29.0304g
Distilled water	4050ml

For 10mM PBS, solutions A and B were mixed together, 87.75g of sodium chloride added, the pH adjusted to pH 7.4 and the volume made up to 10 litre with distilled water. Storage was at RT.

2. TMB#

5mg TMB was dissolved in 0.5ml Dimethylsulphoxide (DMSO) in the dark and added to 50ml sodium acetate/citrate buffer (pH 6.0; see below). Immediately prior to use 36.4µl of hydrogen peroxide (30% v/v) was added to the above solution.

3. SODIUM ACETATE/CITRATE BUFFER (pH 6.0)

0.1M SODIUM ACETATE

0.8204g sodium acetate was dissolved in distilled water and made up to a 100ml.

0.1M CITRIC ACID
2.1014g citric acid was dissolved in distilled water and made up to 100ml.

The initial pH of the 0.1M sodium acetate solution was checked and 3-5ml of the 0.1M citic acid solution was added to a final pH of 6.0. Storage of working aliquots was
at 4°C.

PART B - SOLUTIONS FOR SDS-PAGE

1. BOILING MIX

Stacking gel buffer (see below)	1.0ml
25% SDS+ in distilled water	0.8ml
B-mercaptoethanol	0.5ml
Glycerol	1.0ml
Bromophenol blue (powder)	to colour

+ - Sodium dodecylsulphate polyacrylamide

2. 1% AMMONIUM PERSULPHATE#

0.1g of ammonium persulphate was dissolved in 900µl of distilled water.

3. RESOLVING GEL BUFFER (pH 8.9)

Tris base	36.3g
SDS	0.8g

The chemicals were dissolved in distilled water, the pH adjusted to 8.9 and made up to a volume of 200ml. Working aliquots were stored at 4°C.

4. ACRYLAMIDE/BISACRYLAMIDE FOR RESOLVING GEL SOLUTION

Acrylamide	28.5g
Bisacrylamide	1.5g

The chemicals were dissolved in distilled water, made up to 100ml and filtered through Whatman No. 1 filter paper. Working aliquots were stored at 4°C.

5. ACRYLAMIDE/BISACRYLAMIDE FOR STACKING GEL

Acrylamide	29.26g
Bisacrylamide	0.75g

The chemicals were dissolved in distilled water, made up to 100ml and filtered through Whatman No. 1 filter paper. Working aliquots were stored at 4°C.

6. STACKING GEL BUFFER (pH 6.7)

Tris base	5.9g
SDS	0.4g

The chemicals were dissolved in 80ml distilled water, adjusted to pH 6.7 with concentrated HCl, then made up to a final volume of 100ml with distilled water. Working aliquots were stored at 4°C.

7. MOLECULAR WEIGHT STANDARDS

The protein components of the molecular weight standards are;

PRODUCT NO.	PROTEIN	WEIGHT (Daltons)
A7517	Bovine albumin	66,000
A7642	Egg albumin	45,000
G5262	Glyceraldehyde-3-phosphate dehydrogenase	36,000
C2273	Carbonic anydryrase	29,000
T9011	Bovine trypsinogen	24,000
T9767	Trypsin inhibitor	20,100
L6385	Lactalbumin	14,200

The molecular weight standards were prepared according to the manufacturers instructions (Sigma Chemical Company) and diluted in PBS/boiling mix (2:1 v/v) depending on the concentration of proteins required.

8. TANK BUFFER

Tris base	15.8g
Glycine	10.0g
SDS	2.5g

The chemicals were dissolved in distilled water and made up to 2.5 litres. Storage was at RT.

PART C - SOLUTIONS FOR SILVER STAINING

All solutions for silver staining were stored at RT.

1. FIX

Methanol (analaR)	500ml
Glacial acetic acid	120ml
37% formaldehyde	0.5ml

These solutions were mixed together and made up to 1 litre with distilled water.

2. WASH A (50% Ethanol)

Ethanol	500ml
Distilled water	500ml

3. PRETREAT#

Sodium thiosulphate	0.5g
Distilled water	250ml

4. IMPREGNATE#

Silver nitrate	0.5g
37% formaldehyde	187.5µl
Distilled water	250ml

5. DEVELOP#

Sodium carbonate (anhydrous)	15g
37% formaldehyde	125µl
Sodium thiosulphate	1.0mg
Distilled water	250ml

6. STOP (12% Acetic acid/ 50% Methanol)

Methanol (analaR)	500ml
Glacial acetic acid	120ml
Distilled water	380ml

7. WASH B (50% Methanol)

Methanol (analaR)	500ml
Distilled water	500ml

PART D - SOLUTIONS FOR WESTERN BLOTTING

1. CATHODE BUFFER (pH 9.4)
20mM Tris-HCl, 40mM amino-caproic acid, 20% Methanol

Tris-HCl	0.79g
Amino-caproic acid	1.312g
Methanol (analaR)	50ml

The chemicals were dissolved in the methanol, distilled water added to a volme of 200ml, the pH adjusted to 9.4 and made up to a final volume of 250ml in distilled water. Working aliquots were stored at 4°C.

3. ANODE 2 BUFFER (pH 10.4)
25mM Tris-HCl, 20% Methanol

Tris-HCl	0.9875g
Methanol (analaR)	50ml

The chemical was dissolved in the methanol, distilled water added to a volume of 200ml, the pH adjusted to 10.4 and made up to final volume of 250ml. Storage was as described for Cathode buffer.

4. ANODE 1 BUFFER (pH 10.4)
0.3M Tris-HCl, 20% Methanol

Tris-HCl	11.85g
Methanol (analaR)	50ml

The chemical was dissolved in the methanol, distilled water added to a volume of 200ml, the pH adjusted to 10.4 and made up to a final volume of 250ml. Storage was as described for Cathode buffer.

4. DAB ENHANCED WITH NICKEL CHLORIDE#

60mg DAB was dissolved in 90ml 50mM tris base (pH 7.6; see below). 10ml 0.3% nickel chloride was added and the resultant solution filtered through Whatman No. 1 filter paper. 100µl of hydrogen peroxide was added immediately prior to use.

5. TRIS BASE (50mM)

Tris base	6.057g

Dissoved in distilled water and made up to a volume of 1 litre with distilled water.

PART E - SOLUTIONS FOR MEDIA PREPARATION

All stock solutions for media preparation were stored at -4°C.

1. BENOMYL (selective for basidiomycetes)

A stock solution of benomyl was prepared by dissolving 0.8g benlate in 1 litre of 50% ethanol (4ppm. benomyl). 1ml of this solution was used per 100ml media.

2. STREPTOMYCIN

1ml of a 10% (w/v) Streptomycin stock solution was used per 100ml of media.

PART F - SOLUTIONS FOR PROTEIN ASSAYS

1. 1.0M SODIUM HYDROXIDE

40g sodium hydroxide was dissolved in distilled water and made up to a volume of 1 litre. Appropriate dilutions were prepared using distilled water as the diluent.

PART G - SOLUTIONS FOR MONOCLONAL ANTIBODY PRODUCTION

1. GROWTH MEDIUM

RPMI 1640	100ml
Penicillin/streptomycin (p/s)	1ml
L-glutamine (200mM; Flow)	1ml
sodium pyruvate (100mM; Flow)	1.25ml
FCS	10ml

p/s were both at concentrations of 5,000µg per ml sterile distilled water and were supplied by Flow.

The following chemicals were added to the growth medium when required;

HT(x50)/Growth medium	2ml
HAT(x50)/Growth medium	2ml

2. FREEZING MIXTURE

Dimethylsulphoxide (DMSO) and FCS were diluted in growth medium to concentrations of 10% (v/v) and 50% (v/v) and stored at 4°C.

3. EDTA

To prepare a stock solution, 2g EDTA was dissolved in 100ml distilled water. Phenol red (not more than 0.5ml) was added to show pH (phenol red : yellow pH 6.8 - red pH 8.4). The pH was adjusted to ~7.2 (orange/red) the solution dispensed into 5-10ml aliquots and autoclaved at 121°C/15 minutes. This stock solution was stored at RT. For use, the EDTA stock solution was diluted x100 in Dulbecco's A buffer.

4. DULBECCO'S A (phosphate buffered salts)

analaR sodium chloride	8.0g
analaR potassium chloride	0.2g
analaR disodium hydrogen orthophosphate (anhy)	1.15g
analaR potassium dihydrogen orthophosphate (anhy)	0.2g

The above chemicals were dissolved in sequence in 800ml distilled water, the pH adjusted to 7.3 and the volume made up to 1 litre. 50-100ml aliquots were prepared and autoclaved at 121°C/15 min. (anhy - anhydrous). Storage was at RT.

5. PEG MW 1,300-1,600

PEG was used at a concentration of 50% (w/v) in growth medium. PEG was autoclaved at 121°C/15 min and while hot diluted with growth medium which was heated to 60°C (2g PEG + 3ml growth medium) and adjusted to a pH of 9.0 for use in fusions i.e. 1ml 50% PEG + 1µl 3N NaOH. PEG itself was stored at RT and the above solution prepared fresh for fusions.

6. TRIS BUFFERED SALINE (TBS; pH 7.6)

Tris base	2.42g
Sodium chloride	8.0g
Hydrochloric acid (1M)	3.8ml

Chemicals were dissolved in distilled water and the solution adjusted to pH 7.6. The volume was diluted to 1l with distilled water and stored at RT.

7. TBS-TWEEN (TBS-T)

Tween 20	1ml
TBS	999ml

This solution was thoroughly mixed and stored at RT.

8. SUBSTRATE SOLUTION#

One tablet of 4-chloro-1-naphthol was dissolved in 10ml cold methanol (2-8°C). One drop of hydrogen peroxide (30% v/v) was added to 50ml TBS and mixed with the dissolved tablet immediately prior to use.

APPENDIX 2

PUBLICATIONS

PUBLICATIONS

McDOWELL, H. E., BUTTON, D., AND PALFREYMAN, J. W. (1992). Molecular analysis of the basidiomycete *Coniophora puteana*. Inter. Res. Group on Wood Pres. Doc. No. IRG/WP/1534.

McDOWELL, H. E., AND PALFREYMAN, J. W. (1992). The use of molecular methods to identify wood decay organisms. 2. Analysis of *Coniophora puteana*. Wood Protection. In Press.

PALFREYMAN, J. W., **TIMMONS, H. E.,** VIGROW, A., BUTTON, D., AND KING, B. (1990). Identification of wood decay basidiomycetes using SDS-PAGE. Inter. Res. Group on Wood Pres.

POSTER PRESENTATIONS

Scottish Mycology and Plant Pathology Group Meeting, University of Aberdeen, 1991.
"Identification of the Wet Rot Organism *C. puteana*."

Building Pathology Conference, Trinity College, Oxford, 1991.
"Methods for Identification of *C. puteana*."

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THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Working Group 1a Biological Problems (Flora)

Molecular analysis of the basidiomycete **Coniophora puteana**

by

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ABSTRACT

Sodium dodecylsulphate polyacrylamide gel electrophoresis and Western Blotting, using a polyclonal antiserum produced against a whole cell extract of **Coniophora puteana**, were used to analyse the major proteins and antigens of the wet rot organism **C. puteana**. The macromolecule profiles of this organism were different from other members of the **Coniophora** genus and from a set of unrelated organisms. However the profiles for **C. marmorata** and **C. arida** were more like that of **C. puteana** than other organisms analysed. Analysis, by SDS-PAGE of exoproteins indicated differences between members of the **Coniophora** genus but, whilst there were some intra-species differences overall profiles were similar for all isolates of **C.puteana** tested. Some cross reactivity of the **Coniophora** antiserum was noted, both in Western Blotting and in enzyme immunoassay and, whilst the antiserum was produced against liquid culture grown organism it was able to detect **C.puteana** when extracted from infected wood blocks. Furthermore, unlike some other basidiomycetes analysed serum components did not bind non-specifically to **C. puteana**.

KEYWORDS: **Coniophora puteana**, SDS-PAGE, Western blotting, enzyme immunoassay

INTRODUCTION

Previous studies have indicated the value of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the associated technique of western blotting in the detection and identification of a wide range of microorganisms including the wood decay basidiomycete **Serpula lacrymans** (Vigrow et al, 1989; Schmidt and Kebernik, 1989; Palfreyman et al, 1991). Each of these sets of authors demonstrated that a range of isolates of **S.lacrymans** had a single protein profile and that this profile could be distinguished from that of other organisms tested. In addition Vigrow et al (1991) also showed that the antigenic nature of **S.lacrymans** was unique and that using a combination of a relatively non-specific antiserum and western blotting this organism could be distinguished from a range of other wood decay basidiomycetes that are found to infect timber in buildings.

Studies on other wood decay organisms, e.g. **Poria placenta, Coriolus versicolor, Lentinus lepideus**, etc (Goodell and Jellison, 1986; Palfreyman et al, 1988; Glancy et al, 1990; Kim et al, 1991) have shown the usefulness of molecular, and in particular immunological, techniques in the identification and detection of specific fungi though extensive cross reactivity of many of the immunological reagents produced in these studies may limit their value. To extend molecular analysis to a further basidomycete of importance in the UK, and elsewhere, viz. **C.puteana** we have initiated a study of this organism with a view to understanding its molecular nature and ultimately to produce detection and identification systems.

C.puteana, is the major causative organism of wet rot in the UK. It, and other members of the genus **Coniophora** are distributed widely around the world, its overall distribution being reported by Ginns (1982). In Europe **C.puteana** is one of the most important fungi causing decay of timber in buildings (Cartwright and Findlay, 1958). It is particularly important, in view of the appropriate remedial treatments, to distingush decay caused by this organism from that caused by the dry rot organism **S.lacrymans**. In this preliminary report we present an analysis of the molecular profiles of **C.puteana** and related organisms together with a preliminary analysis of an antiserum produced against a whole cell extract of the organism. Further details of the analysis will be presented elsewhere.

MATERIALS and METHODS

The following organisms were obtained from the Building Research Laboratory, Garston, U.K. for use in this study: **C.puteana** FPRL 11E, FPRL 11A, FPRL 11Q, FPRL 11B; **C.arida** FPRL 411, **C.marmorata** FPRL 410; **S. lacrymans** FPRL 12C; **P.placenta** FPRL 280; **Fibroporia vaillantii** FPRL 149; **Amyloporia xantha** FPRL 62F, **Laetiporus sulphureus** FPRL 29, **Gloeophyllum sepiarium** FPRL 10D and **Heterobasidion annosum** FPRL 41E. A range of other organisms were also included in this study: e.g. **C.puteana** BAM15, **Gloeophyllum trabeum** BAM 109, **Trichoderma harzianium** IMI 206040 and **Verticillium** spp. (isolated at the Forestry Commission, Northern Research Station). **Coniophora** spp. U20 was isolated at Dundee Institute of Technology.

Preparation of fungi for analysis was essentially as described by Palfreyman et al (1991). Organisms were routinely maintained on 5% malt extract/ 2% agar. All material for electrophoretic analysis was grown on 5% malt extract broth and harvested when 75% of the petri dish was covered in mycelium. During harvesting the fungal material was lifted from the broth and the original inoculation core was cut out. The remaining mycelium was washed with ultra pure water until the filtrate ran clear and partially dried on filter paper. The material was then freeze dried prior to storage at -180°C until analysis.

Details of the SDS-PAGE technique used have been previously described in Palfreyman et al (1991). The method is basd on the technique of Laemmli (1970) as modified by Marsden et al (1978). Salient features of the technique include, a) the use of 5-15% polyacrylamide gradient gels, b) the use of a discontinuous buffer system, c) separation of protein species on an LKB 2001 vertical slab gel electrophoresis unit using 35mA/gel at 4°C. (The use of the low temperature, whilst not essential, significantly improves the quality of the protein separations produced.)

To visualise separated proteins, gels were stained using the silver staining technique of Blum et al (1987) as described in Palfreyman et al (1991). Briefly fixed gels were pretreated with sodium thiosulphate, impregnated with silver nitrate and formaldehyde and developed with sodium carbonate, formaldehyde and sodium thiosulphate. Development was stopped with methanol/ acetic acid once an appropriate degree of staining was reached.

Western blotting of proteins separated by SDS-PAGE was undertaken as described by Vigrow et al (1991). Essentially proteins were blotted on to Immobilon (Millipore, UK, Ltd) using the Sartoblot semi-dry electrophoretic transfer system (Sartoblot II, Sartorius Ltd.) following the method of Towbin et al (1979) as modified by Kyhse-Anderson (1984). After transfer, standard protein tracks were stained with India Ink (Hancock and Tsang, 1983). The remainder of the blot was blocked with phosphate buffered saline (PBS) containing Tween 20 and new born calf serum (NCS) (PBS/10% NCS/0.5% Tween 20), incubated with antiserum diluted appropriately and stained with horse radish peroxidase linked anti-rabbit serum followed by diaminobenzidine and nickel chloride (Harlow and Lane 1988). When sufficient colour had developed in blots the reaction was stopped by a final wash with PBS prior to drying.

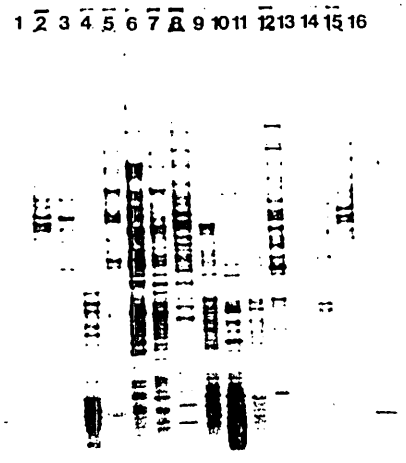
An antiserum to **C.puteana** was developed in a rabbit by methodology to be described elsewhere (McDowell, manuscript in preparation). This antiserum was used in the western blotting procedure described above. For use in enzyme immunoassay (EIA) the antiserum was diluted 1:200 in PBS/5% NCS/0.05% Tween 20. The antigen for the EIA was diluted in PBS and incubated in the wells of a microtitre plate overnight at 4°C. After blocking with PBS/10% NCS/ 0.5% Tween 20 antiserum was added to the wells, incubated for 1h at room temperature, the wells were washed x6 with PBS/0.05% Tween 20 and bound antibodies were detected with HRP linked antirabbit serum followed by tetramethyl benzidine. After 30 min colour development was stopped with 2M sulphuric acid. For the analysis of infected wood blocks small pine blocks ($1cm^3$) were decayed to a variety of weight losses as described for **S.lacrymans** by Palfreyman et al (1991).

RESULTS

To confirm that C.puteana had a molecular profile different from a range of wood decay basidiomycetes and other wood inhabiting organisms SDS-PAGE analysis on extracts of 11 different organisms was carried out and the results are illustrated in Fig.1. As was expected unique profiles for each of the organisms tested was found. The organism whose profile was most similar to that of C.puteana FPRL 11E was isolate U20 (track 3), isolated from a decayed wood sample. It was initially thought that this isolate was C.puteana but as can be seen from Fig. 1 there are a number of differences between this organism and the standard strain of C.puteana used in this study FPRL 11E.

Fig.1

SDS-PAGE analysis of the major protein species found in whole cell extracts of a range of wood decay/inhabiting organisms.

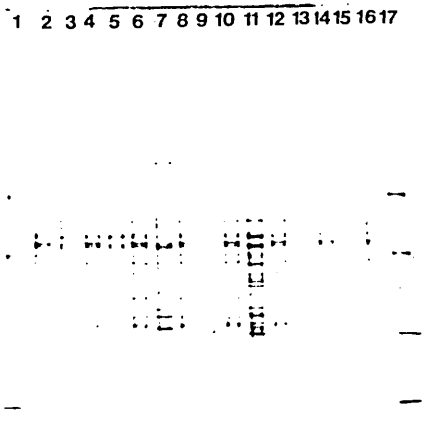


Tracks 1,16 represent standard molecular weight markers ranging in molecular weight from 14,200 to 66,000. Tracks 2,8 and 15 represent extracts of C.puteana FPRL 11E; track 3, Coniophora isolate U20; track 4, S.lacrymans; track 5, P.placenta; track 6, F.vaillantii; track 7, A.xantha; track 9, L.sulphureus; track 10, G.trabeum; track 11, G.sepiarium; track 12, H.annosum; track 13, Verticillium spp.; track 14, T.harzianium.

The profile obtained for C.puteana FPRL 11E could be reproduced from one gel to another and did not alter during culture and subculture of the organism. To compare this standard profile with other isolates of C.puteana and with other members of the Coniophora genus extracts of C.puteana, C.marmorata and C.arida, were analysed and the results are shown in Fig. 2.

Fig.2

Analysis of the major protein species of whole cell extracts of a variety of members of the Coniophora genus.



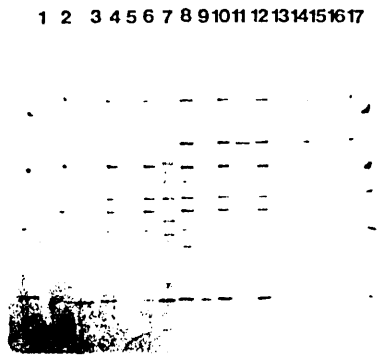
Tracks 1,17 represent standard molecular weight markers (see Fig.1). Tracks 2,4,6,8,10,12,14 and 16 represent C.puteana FPRL 11E; track 3, C.puteana FPRL 11A; track 5, C.puteana FPRL 11B; track 7, C.puteana FPRL 11Q; track 9, C.puteana BAM 15; track 11, Coniophora isolate U20; track 13, C.marmorata FPRL 410; track 15, C.arida FPRL 411.

The data shown in Fig.2 indicate the relative similarity between isolates of the Coniophora genus tested. However differences were apparent between profiles and these differences could perhaps be utilised to identify specific isolates. In particular comparison of tracks 11, 12 and 13 reveals that isolate U20 is more similar to C.marmorata than C.puteana and may indeed be an isolate of the former organism.

All studies reported in Fig.1 and 2, and those reported by Schmidt and Kebernik (1989) and Vigrow et al (1989) have analysed whole cell extracts of fungal microorganisms. However, for the purposes of antiserum production, and specifically to improve the specificity of antibody based reagents, immunogens based on loosely bound surface components have been utilised, e.g Kaufman and Standard (1987), Dewey et al (1989), Kim et al (1991). An analysis of the analogous proteins produced by Coniophora spp. is shown in Fig.3

Fig.3

SDS-PAGE analysis of exoproteins of members of the **Coniophora** genus



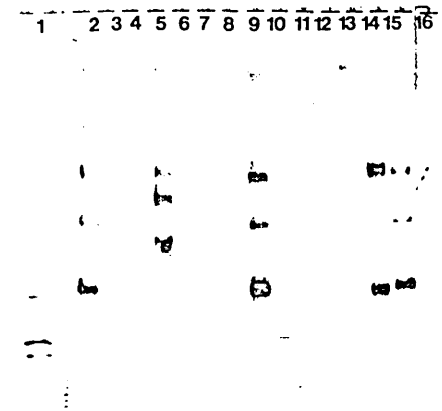
Tracks 1,17 represent standard molecular weight markers. Tracks 2,4,6,8,10,12,14,16, represent exoproteins of **C.puteana** FPRL 11E; track 3, **C.puteana** FPRL 11A; track 5, **C.puteana** FPRL 11B; track 7, **C.puteana** FPRL 11Q; track 9, **C.puteana** BAM 15; track 11, **Coniophora** isolate U20; track 13, **C.marmorata** FPRL 410; track 15, **C.arida** FPRL 411.

Unlike the SDS-PAGE profiles of whole cell extracts of **C.puteana** which show a high degree of similarity, variability in exoprotein profiles is evident in Fig. 3. However a similar general overall pattern for the organisms is detectable and the patterns for **C.puteana** isolates are generally more similar to each other than to isolates of **C.marmorata** and **C.arida** (compare tracks 14, 13 and 15 for example).

Whilst it is possible to identify **C.puteana** isolates using SDS-PAGE simpler *methods for organism* identification and analysis can be based around immunological techniques. To initiate an antigenic analysis of **C.puteana** the antiserum described in **Material and Methods** was tested against various isolates of wood decay and wood inhabiting organisms in both Western blotting (Fig.4) and EIA (Fig.5).

Fig.4

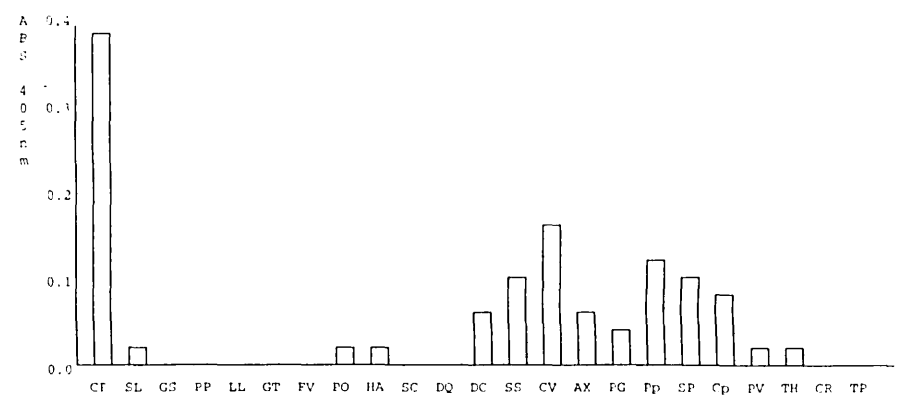
Western blotting analysis of various wood inhabiting microorganisms.



Tracks 1,16 represent standard molecular weight markers stained by the India ink staining method. Tracks 2,9 and 15, **C.puteana** FPRL 11E; track 3, **T.harzianium**, track 4, **Verticillium** spp.; track 5, **H.annosum**; track 6, **G.sepiarium**; track 7, **G.trabeum**; track 8, **L.sulphureus**; track 10, **A.xantha**; track 11, **F.vaillantii**; track 12, **P.placenta**; track 13, **S.lacrymans**; track 14, **Coniophora** isolate U20.C

Fig.5

Cross reactivity of the **C.puteana** antiserum with a range of different organisms.



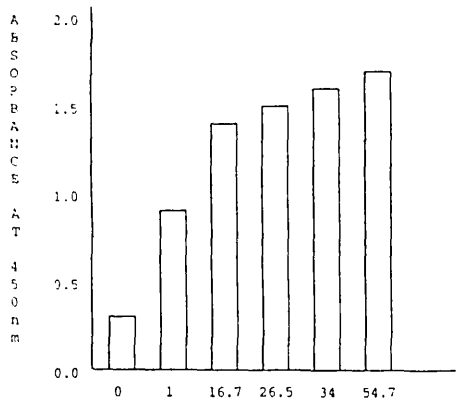
In Fig.5 the following abbreviations have been used: CP (C.puteana), SL (S.lacrymans), GS (G.sepiarium), PP (P.placenta), LL (L.lepideus), GT (G.trabeum), FV (F.vaillantii), PO (Pleurotus ostreatus), HA (H.annosum), SC (Schizophyllum commune), DQ (Daedalea quercina), DC (Daldina concentrica), SS (Stereum sanguinolentum), CV C.versicolor AX (A.xantha), PG (Peniophora gigantea), Pp (Paxillus panuoides), SP (Serpula pinastri), Cp (Ceratocystis picea), PV (Paecilomyces variotti), TH (T.harzianium), CR (Cladosporium resinae), TP (Trichoderma polysporium). Organisms not listed in Materials and Methods were obtained from a variety of sources, mainly B.R.E., Garston, details of organisms will be given elsewhere (McDowell, manuscript in preparation).

As can be seen from Fig. 5 there was a relatively low degree of cross reactivity between the Coniophora antiserum and the range of organisms tested. There was very little reaction between the organism and control sera (data not shown).

To determine if the EIA system used for cross reactivity studies could be used to determine relative levels of C.puteana antigen in extracts of laboratory infected wood small wood blocks inoculated with the organism and incubated for various time periods were freeze dried, ground up and extracted in PBS. Suitably diluted samples were bound on to microtitre plate wells and assayed for antigen as described in the Materials and Methods. Results of the assay are shown in Fig.6. Generally it can be seen that as weight loss increased antigen levels increased similarly.

Fig.6

EIA analysis of extracts of infected wood blocks.



Data in Fig.6 relates weight loss in wood blocks to absorbance in the EIA system detailed in Materials and Methods.

DISCUSSION

The data given in this paper represent an initial molecular analysis of the major causative organism of wet rot in the UK, viz. C.puteana. Despite the great importance of this fungus in terms of the financial losses associated with its action few studies are undertaken on the organism no doubt because its control is relatively simple, at least compared to the dry rot fungus S.lacrymans. Its importance as a decay fungus is however recognised by its inclusion as a standard organism in EN113 testing.

The results presented in this paper indicate that C.puteana has a unique molecular structure in terms of both its SDS-PAGE protein pattern and its antigenic nature. Either SDS-PAGE or Western blotting can be used to identify the organism when cultured in the laboratory, however its identification from infected field samples may, as also applies to S.lacrymans, be best effected by analysis of western blotting profiles (unpublished observations). The appearance of unique protein patterns at both species and genus level allows easy differentiation between of members of the Coniophora genus and the identification of new isolates, e.g. U20.

Previous studies on the molecular nature of wood decay fungi (Schmidt and Kebernik, 1989; Vigrow et al, 1989) have analysed whole cell extracts of organisms as it was thought that preparations made in this way would be the most likely to produce similar profiles for related organisms. An analysis of the exoprotein profiles of various isolates of Coniophora confirms this hypothesis. Whilst similar profiles are found for related organisms the differences perceived are greater than for whole cell extracts. That there are, however, great similarities between profiles suggests that the use of exoproteins as immunogens for the production of either polyclonal (e.g. Kim et al, 1991) or monoclonal antibodies (e.g. Dewey et al, 1989) will produce useful immunological reagents. Reagents which will react with a range of isolates of any particular organism.

Whilst these molecular techniques represent relatively sophisticated methodologies for the identification of organisms, simple enzyme immunoassays represent more readily applicable diagnostic tools, as reported by Goodell and Jellison (1986), Breuil et al (1988), Palfreyman et al (1988) and Kim et al (1991). In this paper a simple EIA for C.puteana is reported based on the production of an antiserum against a whole cell immunogen. Whilst this assay shows limited specificity for C.puteana it can detect the organism when present in both liquid and wood cultures. Attempts to improve specificity of the EIA by use of an exoprotein induced antiserum, c.f. Kim et al (1991), have had little effect on specificity (McDowell, PhD thesis in preparation). The specificity of antisera to wood decay fungi appears to be rather variable, for example, in our studies antisera to S.lacrymans show a very high degree of cross reactivity in Western blotting (Vigrow et al, 1991) compared to the cross reactivity found for the Coniophora antiserum. In a study on exoantigen antisera to a range of different wood decay fungi Kim et al (1991) reported a similar finding with antiserum to P.placenta being highly cross reactive, whereas an antiserum to Tyromyces palustris showed relatively low cross reactivity. In marked contrast to studies on S.lacrymans (Palfreyman et al, 1988) there was little reaction between Coniophora spp. and control sera.

The data presented here indicate the usefulnes of molecular and immunological methods for the identification and detection of an important wood decay fungus. However, whilst sophisticated techniques can produce satisfactory diagnostic tools the development of simpler methods requires the generation and validation of more specific immunological reagents.

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The use of molecular methods to identify wood decay organsims. 2. Analysis of *Coniophora puteana*.
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ABSTRACT

Molecular analysis, by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), of one of the causative organisms of wet rot, *Coniophora puteana*, indicates that there is substantial similarity in protein profiles between strains of this fungus and between the different species of *Coniophora* tested. These profiles do not alter significantly when the fungus is grown on a variety of media, including wood. Analysis by SDS-PAGE of other organisms associated with decay indicates great variety in protein profiles. Western blotting studies using an antiserum raised against *C. puteana* FPRL 11E, indicate that the members of the *Coniophora* species tested have similar, though distinguishable antigenic profiles to one another, that the overall profile is unique to the genus and that the profile is not altered when the fungus is grown in wood. This simple method of identification amplifies the fine molecular differences between the species of *Coniophora* and strains of *C. puteana* and suggests that reclassification of some standard strains of *C. puteana* may be necessary.

IN PRESS

THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Working Group I a Biological problems (Flora)

Identification of wood decay basidiomycetes using SDS-PAGE

by

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SUMMARY

The technique of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) coupled with silver staining has been used to produce protein profiles of a range of different wood decay basidiomycetes. Comparison of inter-isolate differences with inter-species differences has facilitated the production of a similarity index which allows the identification of unknown isolates using SDS-PAGE. For example, when grown under identical conditions all isolates of Serpula lacrymans had a simlarity index of >40% whilst all other wood rotting organisms tested had indices of <35%.

To further evaluate the technique, protein profiles of a number of different isolates of basidiomycete organisms have compared against standard preparations of known fungi. Isolates of some organisms obtained from the historic ships the Frigate Unicorn and the RRS Discovery have been identified by this technique.

KEYWORDS : SDS-PAGE, Serpula lacrymans, identification, similarity index

INTRODUCTION

Identification of wood decay fungi is normally undertaken by observation and characterisation of fruiting bodies or by detailed knowledge of environmental conditions surrounding an outbreak of a particular organism. Experience plus use of reference texts such as Cartwright and Findlay (1958) or Bravery et al (1987) leads to correct identification in most cases however even expert surveyors disagree in some cases. As part of a general research programme into the use of molecular methods to identify decay fungi we have investigated the potential use of SDS-PAGE as a method for the confirmation of fungal identity (Vigrow et al 1989). Initial results indicated that a range of S.lacrymans isolates tested possesed similar profiles on SDS-PAGE, a result confirmed by other workers (Schmidt and Kebernik 1989).

Studies also indicated that other decay organisms produced somewhat different profiles on SDS-PAGE and suggested that this technology could be used for identification purposes as it has in other areas of microbial taxonomy (Jackman et al 1983, Kersters 1985). To test this hypothesis we have undertaken a detailed analysis of a range of basidiomycete fungi and the results of our study are reported in this paper. Further details of the methodology will be reported elsewhere (Palfreyman et al, submitted for publication).

MATERIALS AND METHODS

Fungal microorganisms

Most organisms used in this study were obtained from the Building Research Laboratory, Garston, U.K., the exceptions being the various isolates of S.lacrymans, which were obtained from Dr Bryan Hegarty, Paecilomyces variotti (isolated at Dundee Institute of Technology) and Cladosporium resinae (BM-13388-1-22A), Trichoderma polysporium (IMI 206039) and Gloeophyllum trabeum (BAM(EDW)109) which were obtained from culture collections elsewhere. Samples for identification (see below) were obtained from two historic ships present in Dundee (the Frigate Unicorn and the RRS Discovery). We are grateful to the Unicorn Preservation Society and Dundee Industrial Heritage Limited for allowing us access to the Unicorn and the Discovery respectively.

Preparation of samples for analysis

Preparation of samples for analysis and their investigation by SDS-PAGE has been detailed elsewhere (Vigrow et al 1989).

Percentage similarity index

The relatedness of fungal isolates to the type strain of S.lacrymans (FPRL 12C) used in these studies was determined by the estimation of a similarity index based on the number of bands common to the type strain and any other isolate/ fungal species tested. To estimate the index the total number of common and dissimilar bands between FPRL 12C and the test isolate was counted and the percentage of similar bands termed the percentage similarity index. Indices were only estimated for organisms if more than 20 bands could be identified in the test organism. In most analyses many more than 20 bands were detected. In some instances comparisons with isolates other than FPRL 12C were made. To assist in the estimation of similarity indices graphical representations of silver stained SDS-PAGE gels were made in some cases.

Isolation of organisms

Isolation of organisms from wood samples, and growth of organisms from fruiting body material, was undertaken using standard isolation procedures with benomyl as the selection agent (Hunt and Cobb 1971).

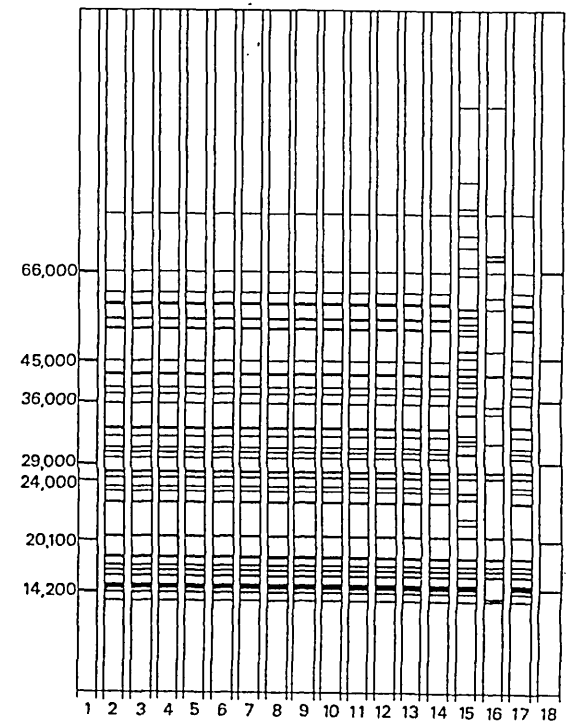
RESULTS

Comparison of banding patterns (molecular profiles) are given for a range of S.lacrymans isolates in Fig. 1. Most isolates gave profiles very similar to the type organism the exceptions being BF-050 and BF-015B. Estimation of the similarity index for isolates other than these two gave values of > 95%, the values obtained for BF-050 and BF-015B were 57.1 and 47.5 respectively.

SDS-PAGE analysis of a range of different wood decay fungi is given in Fig 2. Similarity indices for these and other wood inhabiting organisms are shown in table 1, values vary from 0% for Daedalea quercina to 30.8% for Poria incrassata. None of the organisms gave similarity indices in the range found for the S.lacrymans isolates tested.

Fig 1

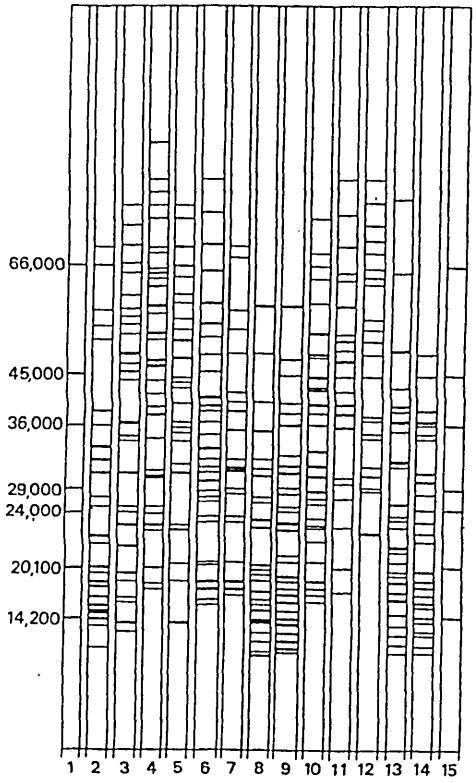
Analysis of isolates of S. lacrymans. 15 isolates of S. lacrymans, including the type strain FPRL 12C (tracks 2 and 17) were analysed by SDS-PAGE. Most isolates gave protein profiles very similar to S. lacrymans FPRL 12C, the two exceptions being BF-050 (track 15) and BF-015B (track 16). Further details of the isolates analysed in this figure will be given elsewhere (Palfreyman et al submitted for publication). Tracks 1 and 18 represent profiles for a standard preparation of molecular weight markers.



Confirmation of the consistency of molecular analysis for particular species comes from comparison of 5 strains of Coniophora puteana which produced very similar protein profiles, likewise 4 strains of the forest pathogen Heterobasidion annosum all had very similar protein profiles when analysed by SDS-PAGE. Furthermore a number of new isolates of S.lacrymans from local building timbers, and isolates of Gloeophyllum trabeum from experimentally infected wood samples, have also been analysed by SDS-PAGE and profiles obtained are similar to the type organism in each case (data not shown).

Fig 2

Analysis of wood decay basidiomycetes by SDS-PAGE. 13 different basidiomycetes were analysed by SDS-PAGE and the results are illustrated in this figure. Tracks 1 and 15 represent molecular weight markers, track 2 is the type strain of S. lacrymans FPRL 12C, other tracks represent the following C. puteana (3), Fibroporia vaillantii (4), Paxillus pannuoides (5), Amyloporia xantha (6), Serpula pinastri (7), Lentinus lepideus (8), Gloeophyllum sepiarium (9), Peniophora gigantea (10), Postia placenta (11), Daedalea quercina (12), Coriolus versicolor (13), and Daldina concentrica (14). Full details of all these organisms will be published elsewhere (Palfreyman et al submitted for publication).



A comparison of the similarity indices for a range of wood inhabiting organisms was made and the results are shown in Table 1. In this table the percentage similarity for 21 different wood inhabiting organisms is compared. In each case the similarity index relates the percentage common bands between any particular organism and S. lacrymans FPRL 12C.

Table 1

Organism	% similarity	Organism	% similarity
Amyloporia xantha	20.9	Paxillus panuoides	21.3
Coniophora puteana	18.5	Peniophora gigantea	10.7
Cladosporium resinae	31.2	Pleurotus ostreatus	15.2
Coriolus versicolor	17.1	Poria incrassata	30.8
Daldina concentrica	19.2	Postia placenta	15.6
Daedalea quercina	0.0	Schizophyllum commune	18.4
Fibroporia vaillantii	19.2	Serpula pinastri	25.0
Gloeophyllum sepiarium	19.8	Stereum sanguinolentum	26.3
Gloeophyllum trabeum	24.1	Trichoderma polysporium	20.7
Heterobasidion annosum	21.5	Lentinus lepideus	16.0
Paecilomyces variotti	15.8		

Identification, by molecular analysis, has also been applied to a range of basidiomycetes isolated from the timbers of the Frigate Unicorn and the RRS Discovery. To date 18 fungi have been isolated from the two ships by plating out on benomyl agar. The identity of the isolates as basidiomycetes has been confirmed morphologically, i.e. by the presence of clamp connections. Analysis of these isolates by SDS-PAGE has been undertaken and the results to date are shown in Tables 2(a) and 2(b). Overall 5 fungi have been positively identified. A further 5 have resemblances to the type fungi used as standards though their identity cannot be positively confirmed, since the similarity indices for these organisms are relatively low. The molecular profiles for the remaining 8 fungi bear little resemblance to any of the type fungi tested.

An example of the analytical procedure used is given in Fig. 3 where the molecular profiles of 9 basidiomycetes isolated from either the Unicorn or the Discovery are compared with the profiles for 7 standard basidiomycete strains. The similarity in banding patterns for isolates in tracks 5,6 and 7 should be noted. The basidiomycete Postia placenta (track 16) shows a similar profile. Similarly the isolate in track 10 (from the Discovery) has a similar profile to the standard basidiomycete Laetiporus suphureus shown in track 15. Confirmation of the identity of specific isolates is always confirmed by repeat gels. In these gels the test isolate is run in tracks next to the standard basidiomycete which it most resembles.

Table 2

Molecular analysis of isolates from the Unicorn and the Discovery.

a) Comparison of isolates

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
A	+																	
B		+																
C			+															
D				+		+	+											
E					+			+	+	+								
F				+		+	+											
G				+		+	+											
H					+			+	+	+								
I					+			+	+	+								
J					+			+	+	+								
K											+							
L												+						
M													+					
N														+				
O															+			
P																+	+	
Q																+	+	
R																		+

+ represents identical SDS-PAGE patterns for specific isolates

For both Table 2(a) and 2(b):

A to R represent the 18 different fungal isolates tested

For Table 2(b) (see next page):

1-15 represent the following standard basidiomycetes. Further details of the actual strains used are given in Vigrow et al (1989) and Palfreyman et al (1990) (submitted for publication).

1. C.puteana
2. S.lacrymans
3. F.vaillantii
4. P.placenta
5. P.panuoides
6. A.xantha
7. S.pinastri
8. L.lepideus
9. P.incrassata
10. P.gigantea
11. D.quercina
12. G.sepiarium
13. G.trabeum
14. P.ostreatus
15. L.sulphureus

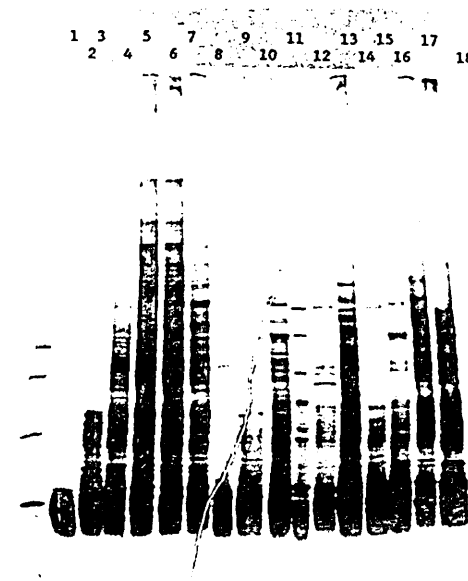
b) Comparison of isolates with various wood decay fungi

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A															?
B															?
C		*	*												
D			+												
E		*	*	*		*									
F			+												
G			+												
H		*	*	*		*									
I		*	*	*		*									
J		*	*	*		*									
K															?
L															?
M															?
N															?
O	+														
P															?
Q															?
R														+	

\+ fungal isolate identical to type strain
* fungal isolate has similarites to type strain
? no identification possible at this stage

Fig 3

Analysis of isolates from the historic ships. Tracks 1 and 18 are molecular weight markers. Tracks 2-10 represent the following isolates from the historic ships, A,B,C,G,D,F,E,H and R respectively. Tracks 11-17 represent extracts from the basidiomycete G.trabeum, G.sepiarium, P.gigantea, S.pinastri, L.sulphureus, P.placenta and P.ostreatus respectively.



DISCUSSION

The studies reported here, together with those published elsewhere (Vigrow et al 1989), have indicated that decay organisms possess unique molecular profiles as determined by SDS-PAGE. They further suggest that this method of analysis can contribute to the identification of basidiomycete fungi as an adjunct to the normal taxonomic methods using complex keys (e.g. Nobles 1965).

Construction of a similarity index has allowed a relatively simple method for quantifying the differences in banding patterns found between S. lacrymans isolates and other fungal species. Two aspects of the similarity index should, however, be stressed. First, it is not comparable as an objective (1985). However, in common with these systems, which can be adjusted for alteration in gel running profiles found in particular experiments, the similarity index can, in the hands of an experienced analyst, be adjusted for minor variations in experimental procedures. Second, initial analysis of gel patterns simply by visual observation is likely to provide, in most cases, an objective assessment of the identity of an organism as accurate as the similarity index. In all cases that we have analysed to date, with the possible exception of S.lacrymans, BF-015B, our initial impressions of the identity of an organism been confirmed by the similarity index measurement. Extension of similarity indices as comparative tests is currently being undertaken for isolates of other organisms.

Application of SDS-PAGE to known isolates of S. lacrymans has yielded useful methodologies and isolates of other decay organisms such as C.puteana and H.annosum (Daniel Galbraith, personal communication) have now been shown to have consistently similar molecular profiles. These results further support the contention that SDS-PAGE may have a role to play in basidiomycete identification. However, applications of the methodology to the identification of unknown isolates from the Unicorn and the Discovery has to date not led to the positive identification of all isolates. A number of isolates have been positively or partially identified by molecular profiles. However the majority of the isolates, as yet, remain unidentified despite their comparison with a wide range of possible fungal species. Furthermore the, as yet, unidentified species do not resemble each other, indicating that at least a further 7 species of organisms remain to be identified.

A number of possible explanations exist for the difficulty in determining the identity of the remaining isolates. First, individual isolates from the same organism may differ in molecular profile and/or second, the range of fungi inhabiting structures such as the ships under study may be greater than in the normal decay situation. Our experience with S. lacrymans, C. puteana and H. annosum together with data from other fields such as analysis of Phytophthora spp. (Hansen et al 1986) would suggest that isolates of the same species will give the same, or very similar profiles. Therefore the first explanation is unlikely to be correct. It seems more probable that a wider range of decay fungi are inhabiting the ships that might be expected by comparison with building timbers. Whilst it is appreciated that some of the basidiomycetes might be of marine origin this seems unlikely due to the isolation procedures used and to the relatively low salinity of the moisture in the wood of the ships. Furthermore only 4 filamentous basidiomycetes have been isolated from the marine environment (Kohlmeyer and Kohlmeyer 1979). It is possible that the unidentified fungi have been mis-classified as basidiomycetes however they all grew on benomyl agar widely used as a selective media for basidiomycetes (Hunt and Cobb 1971) and they all had a 'fluffy white' mycelial appearance and clamp connections consistent with their being members of the basidiomycotina. We are currently increasing the range of organisms used for comparative purposes with a view to positively identifying the remaining isolates.

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